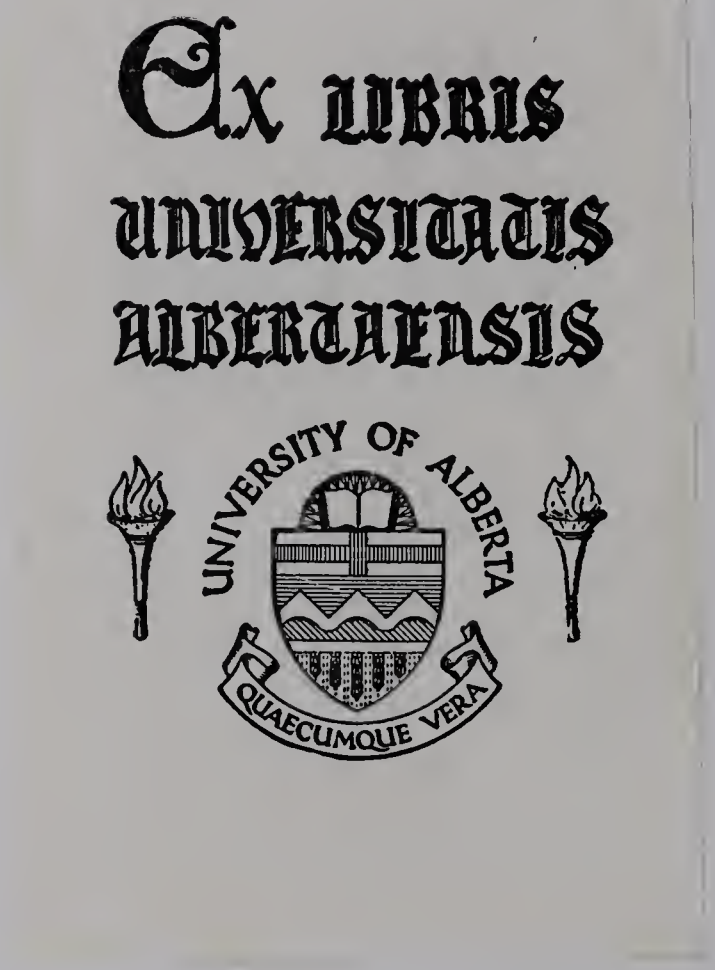

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CERTAIN ASPECTS OF THE METABOLISM OF SOME QUINOLIZIDINE ALKALOIDS BY *LUPINUS LUTEUS* VAR. *ROMULUS*

by

Donald M. Graham

A THESIS

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OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "CERTAIN ASPECTS OF THE METABOLISM OF SOME QUINOLIZIDINE ALKALOIDS BY LUPINUS LUTEUS VAR. ROMULUS", submitted by Donald M. Graham in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Until recently, alkaloid metabolism has been a comparatively neglected area of plant physiology. This is particularly true in regard to subcellular localization of metabolic reactions, nearly all investigations reported to date having been carried out *in vivo*. Furthermore, microanalytical techniques for alkaloids have lagged behind those for other biochemical compounds, such as, for example, amino acids.

Consequently, a new method was developed for analysis of micromole quantities of lupine alkaloids in plant tissues. The method involves extraction of the tissue with water, buffering the extract, and adsorbing the alkaloids from the solution on a cation exchange resin. The alkaloid mixture is eluted from the resin with acid and the alkaloids are separated from one another by paper chromatography. The individual alkaloid spots are then eluted from the paper and estimated nephelometrically.

This procedure was applied to serial analyses of seeds and seedlings of *Lupinus luteus* var. *Romulus* during and immediately following germination. The ontogenesis of the alkaloids was compared with that of the free amino acids of the plant, and both were correlated with the growth rate of the seedlings.

Stem slices and chloroplasts were tested for metabolic activity towards some quinolizidine alkaloids in an attempt to find an *in vitro* system for simplification of metabolic studies.

Chloroplasts were found to be more active, so they were isolated from seedlings at a stage that appeared, from the studies on the ontogenesis of the alkaloids, to be particularly active metabolically. The chloroplasts were incubated with various quinolizidine alkaloids and the products to which the alkaloids were converted were examined. One particularly obvious conversion, that of sparteine to another, unidentified, alkaloid, was studied in more detail. In particular, it was found that autoclaved chloroplasts were just as active in this conversion as those that had been freshly prepared. Further investigation of this reaction revealed that the factor involved in this conversion was a sugar, and that a variety of carbohydrates, particularly those of low molecular weight, would mediate the reaction. Although some of its properties have been determined, the exact nature of this reaction is not known; nor is the chemical nature of the product, although it behaves in certain respects like an alkaloid.

ACKNOWLEDGMENTS

First and foremost I wish to express my deep gratitude to my supervisor, Dr. M.S. Spencer, for an incalculable amount of gently administered advice, constructive criticism, and help in too many ways to enumerate, throughout the course of this investigation. I should also like to thank those members of the Biochemistry Department who have assisted in one way or another at various times, particularly Dr. B.G. Lane for his interest in this project and for allowing me the use of some of his instruments, sometimes at very short notice; Dr. L.B. Smillie for allowing me the use of his amino acid analyzer on a number of occasions; and Dr. H.B. Collier for his continued interest throughout the course of this investigation. I particularly wish to thank Dr. W.A. Ayer of the Chemistry Department for the time he has devoted to assisting me with problems in organic chemistry, especially in regard to instrumental analysis and interpretation of spectra.

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I also wish to record my thanks to the National Research Council for financial assistance, and particular thanks go to Mrs. A. Kmech who undertook, on short notice, the unenviable task of converting my manuscript into a presentable form.

LIST OF ABBREVIATIONS

HCl Bu-Tol	...	Chromatographic solvent system described by Reifer et al. (59): equilibration is carried out with the lower phase from concentrated HCl-butanol-water (3:4:9); development is with the upper phase of this mixture after the lower phase has been removed and 1 volume toluene added.
t-Am HOAc	....	Chromatographic solvent system used by equilibrating with lower phase of tertiary amyl alcohol-acetic acid-water (1:4:7) and developing with the second.
Sp	.....	sparteine
Li	.....	lupinine
La	.....	lupanine
HOLa	.....	hydroxylupanine
OLa	.....	oxolupanine
Cad	.....	cadaverine
Pip	.....	pipecolic acid
Tri-p	.....	tripiperideine
R_{sp}	.....	R_f of substance in question divided by R_f of sparteine.

A NOTE ON TERMINOLOGY

The terms lupin(e) alkaloid and quinolizidine alkaloid are frequently used interchangeably in the literature. In practice this makes very little difference, although there are a few alkaloids that fit into one category but not into the other. However, in deference to established practice in the biochemical literature, the term lupine alkaloid will be used preferably in this report, but in certain places where the circumstances seem to warrant it, the term quinolizidine alkaloid will be used instead.

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I. INTRODUCTION

Ia. GENERAL INTRODUCTION

A number of reviews and books are available on various aspects of the subject of alkaloids, some of the reviews being very detailed and comprehensive. The Alkaloids (41), by Manske and Holmes, is a very useful reference, particularly on the chemical aspects, and is kept up to date by the addition of supplemental volumes from time to time. However, no integrated general introduction to the subject as a whole, giving principles rather than details, and avoiding undue emphasis on any one group of alkaloids, has yet appeared. Consequently this General Introduction is somewhat more extensive than is customary in a dissertation of this nature. It is not intended to take the place of a full review, but is included to provide a minimal background for a better understanding of the problems discussed in the more specialized introduction devoted to the lupine alkaloids, and of the thesis as a whole.

1. History

Alkaloids are the active ingredients of a great number of pharmacologically-active plants and plant products. These materials have probably been used by man since long before the days of written history and references to them are made in some Egyptian hieroglyphics dating back as far as 3,700 B.C. (31).

Much later, around 900 B.C., Homer refers in the Odyssey to the power of opium to assuage grief. The execution of Socrates by hemlock poisoning is well known.

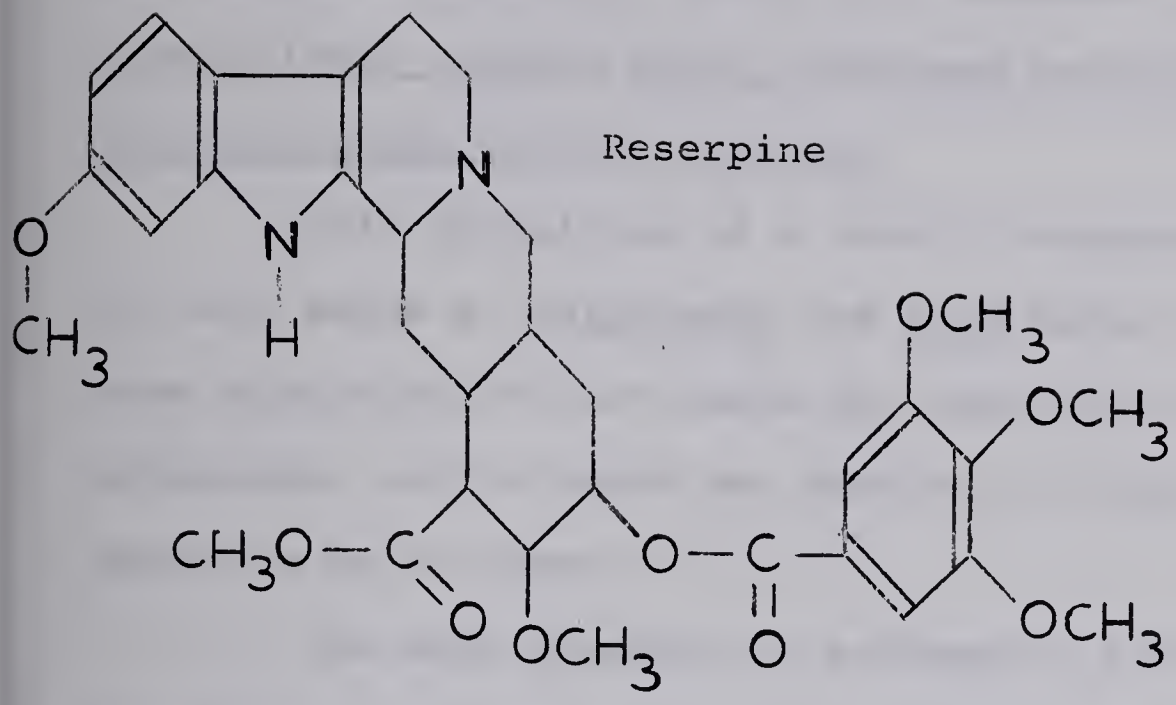
As written records become more abundant, references to various alkaloid products (although their nature was still unknown) appear more frequently, but it was not until A.D. 1817 that any alkaloid was obtained in pure form. In that year Sertürner (80) crystallized morphine (Figure 1) from opium and soon after this many other alkaloids were purified in rapid succession, both by Sertürner himself and by a large number of other workers, notably Pelletier.

Ladenburg, in 1889, was the first to confirm the structure suspected for an alkaloid (coniine) by synthesis (30).

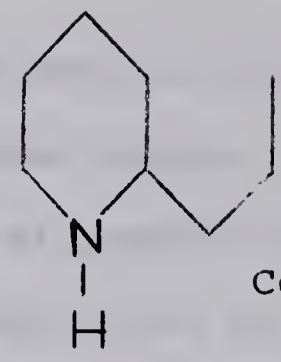
Since that time an enormous volume of research has been done on the chemistry, pharmacology, and physiology of alkaloids. The great variety in their chemical structures has provided the structural and synthetic organic chemist with a huge fund of substances for investigation; their varied and often violent physiological effects on animals have prompted their intense investigation by the pharmacologist; and their wide distribution without apparent function in the plant has aroused interest in the fields of plant physiology and biochemistry. Thus they have an appeal to investigators in a wide variety of research areas. It is in the last-named of these that the least progress has been made, but within the last decade or so an increasingly large number of investigations has been devoted to these aspects of the study of alkaloids.

2. The nature of alkaloids

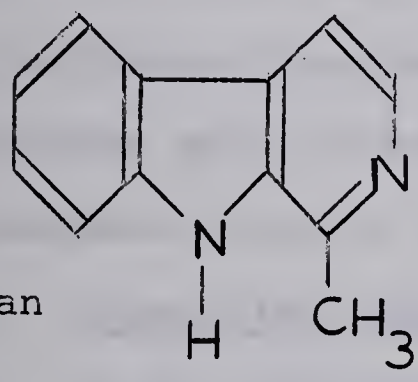
The accepted connotation of the term alkaloid has undergone a number of changes since it was introduced. At first it referred to all naturally-occurring organic bases; then Königs (29) suggested



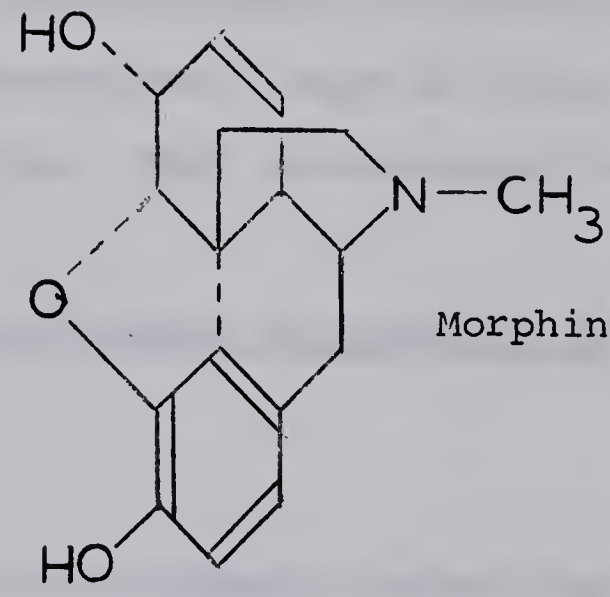
Reserpine



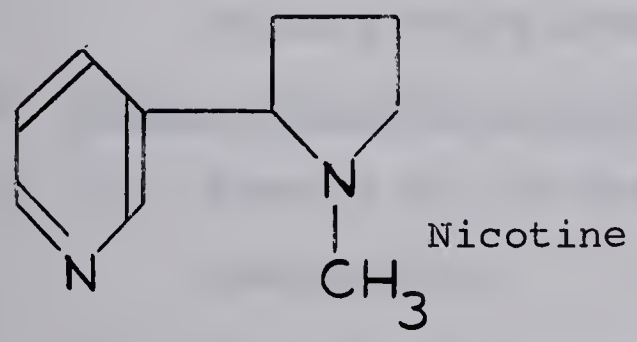
Coniine



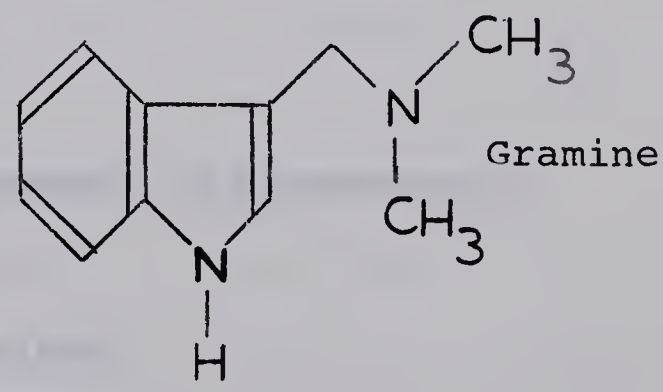
Harman



Morphine

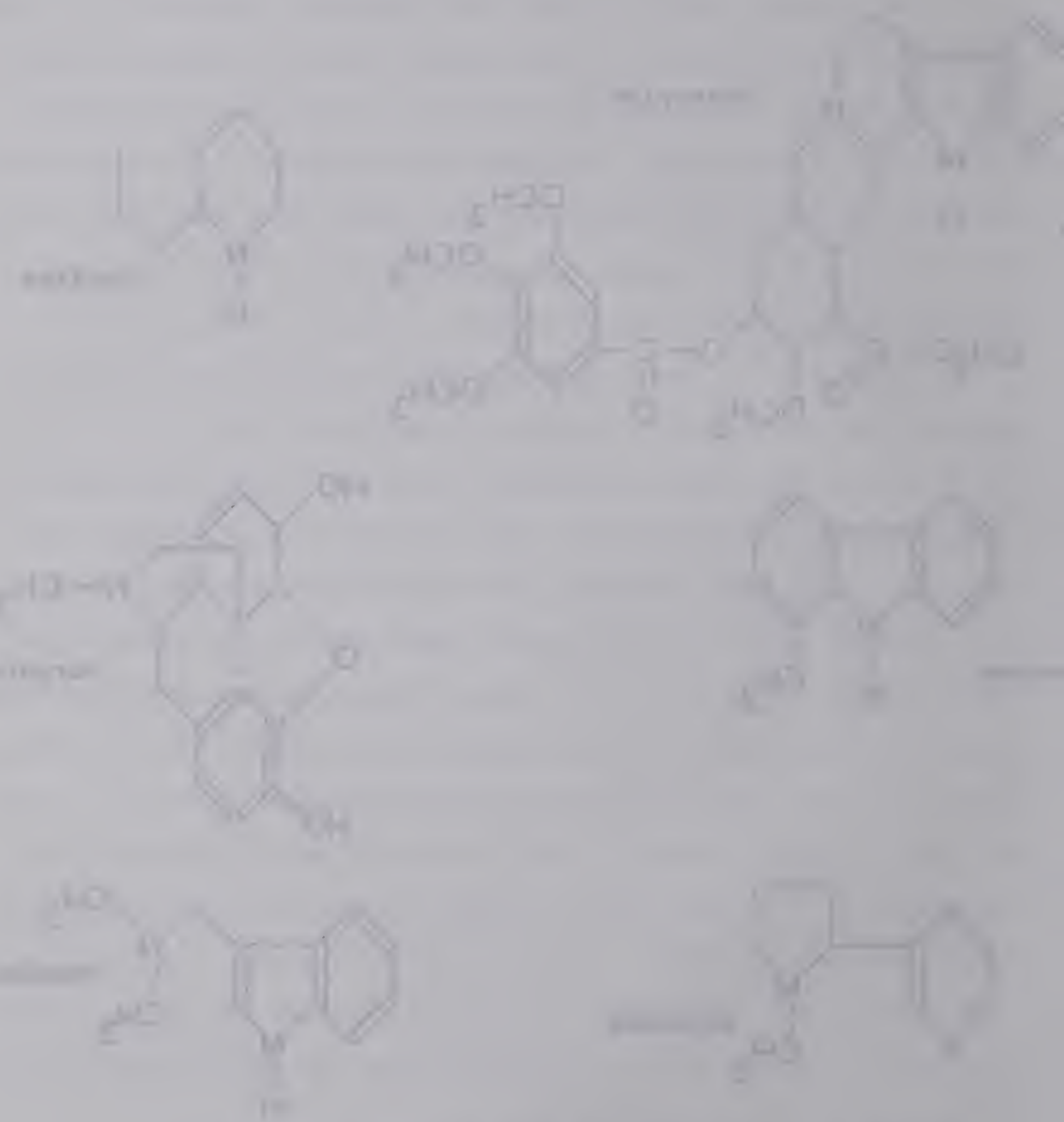


Nicotine



Gramine

Figure 1. Structures of some typical alkaloids.



restricting the term to pyridine derivatives, but this was clearly too narrow a meaning. Henry (23) suggests the following definition: A relatively complex basic substance occurring naturally, and possessing some physiological activity.

This definition is a fairly comprehensive one but the inclusion of such words as relatively and some makes it rather vague. Furthermore some alkaloids are not basic and some basic natural products are not alkaloids; nor is there any mention in Henry's definition of the presence of nitrogen.

The best approach is perhaps to list those definitive properties that have been variously suggested as characteristic of alkaloids and to choose from them according to our preferences. Most of these properties will be found in the following list. They are arranged in approximate order of importance:

A. Properties that are essential if the substance in question is to be considered an alkaloid:
   i. Natural occurrence;
   ii. Presence of organically-bound nitrogen usually other than in primary amine groups;

B. Highly desirable properties:
   i. Some or all of the nitrogen present in heterocyclic combination;
   ii. Distribution among species limited;
   iii. Soluble in water or dilute acid;

C. Optional properties:
   i. Exhibition of physiological effects on animals;

ii. Apparently functionless in the physiology of the plant;

iii. Nonoccurrence in the animal kingdom;

iv. Organic skeleton different from any compound with a known physiological function.

Some of these require further explanation or comment. Non-occurrence in the animal kingdom is mentioned in order to exclude such substances as adrenalin, noradrenalin, choline, betaines, and various other hormones with central nervous, sympatho-, or parasympathomimetic action. This same criterion, however, also has the disadvantage of excluding such substances as samandarine, from the salamanders Salamandra maculosa and S. atra (91), and certain of the toad poisons, which are common both to toads and to some plants (1). The limitation on species distribution excludes such phytohormones as indoleacetic acid. In our present state of knowledge, this requirement is empirically coexistent with lack of known physiological function. If, however, some alkaloid satisfying the limited distribution criterion is later found to have a well-defined function, there is a possibility that it may cease to be classified as an alkaloid - unless all the other alkaloids are found to have similar functions in the species in which they occur. At present, no alkaloid is known to have a function in the plant (although many possible functions have been postulated), and some investigators may choose to regard this apparent lack of function as a defining criterion. A possible new approach to the classification of alkaloids and certain other natural products is discussed in the next sub-section.

3. The function of alkaloids

At the time of writing, none of the substances commonly regarded as alkaloids has been unequivocally shown to possess any physiological function in the plant, although there has long been a great deal of speculation on this problem. For general reviews of this subject see Dawson (15), Marion (42), Mothes (47, 48, 49), and Wenkert (88). Frankenburg (20) also gives a brief summary of this question.

Some of the hypotheses that have been postulated for possible function of alkaloids are listed below:

(a) The alkaloids are end-products of amino-acid metabolism (56);

(b) They are substitutes for inorganic soil cations used for neutralization of organic acids produced in the plant (38);

(c) They are end-products of carbohydrate metabolism (88);

(d) They are a store of reserve nitrogen (91);

(e) They possess coenzyme activity (15, 20);

(f) They act as nonenzymic catalysts (20);

(g) They possess hormone activity (20);

(h) They act as antiauxins (47);

(i) They are end-products of detoxication mechanisms (20);

(j) They offer protection against herbivorous animals (23);

(k) They are leached into the soil where they act as ecologically important factors (15);

(l) They have different, specific functions in different species;

(m) They are vestigial;

(n) They have no function whatsoever.

Objections have been raised against all of these many times, and indeed, no convincing case has ever been put up for any of them, except possibly the last one or two, but it is difficult to devise a direct approach to the problem of assigning a function to any substance; a physiological function is usually discovered first, when a process already being investigated is found to require the presence of some substance which is later identified. The converse process is much more difficult and attempts to assign functionshave usually involved observing correlations between alkaloid content and various physiological parameters. For each such correlation reported for an alkaloid a conflicting one can always be found.

For example, Dawson (15) reports that he has obtained tobacco plants with alkaloid content ranging from extreme values of 12 or 14% of wet weight down to none at all, with no concomitant change whatsoever apparent in any other factor. Mothes (47), on the other hand, claims that it is impossible to breed varieties of tobacco species with absolutely no alkaloid, and that trace quantities always remain; on this ground he suggests that alkaloids do possess some indispensable function in the plant, although he does not venture to suggest what this function might be.

The possibility that alkaloids might possess no function at all is an interesting one, and if true would be enormously difficult to prove - as is any negative hypothesis. I may even be logically impossible to prove. There is no known evolutionary mechanism that can give rise to the widespread formation of functionless substances unless they are obligatory by-products of more important reactions (93).

On the other hand the decidedly equivocal and frequently contradictory results that have been reported between alkaloid content and the physiology of the plant, as well as the total lack of success in attempts to establish firmly any physiological role for these substances is an indication that a wrong approach is being used in the search for functions. The possibility exists that the alkaloids are not indispensable, but this does not mean that they are of no value. What most investigators seem to have overlooked is the possibility that alkaloids may have little value in the ontogeny of the plant, but may well be of great phylogenetic importance.

In the list of postulated functions given above, only two are phylogenetic or partly so - (j) and (k) (they offer protection against herbivores, and they act as ecologically important factors when leached into the soil). An inherited trait requires only a very slight selective advantage in order to become widespread in a relatively small number of generations after it first appears. Thus, to have served a phylogenetic function, the alkaloids need not have conferred any very great increase in probability of survival to maturity in the first plants to have formed them.

Mothes' suggestion that the alkaloids may be leached into the soil as ecologically important factors still appears to be unexplored, although it is known that certain desert plants survive in their harsh environment partly through the medium of mechanisms involving infiltration of the soil in their vicinity with germination inhibitors. Most alkaloid-containing species, however, seem to accumulate these

products in terminal tissues, whether they are synthesized there or formed somewhere else and reach these portions of the plant by translocation. In many species the alkaloids accumulate in the seeds, so that any leaching will be from these rather than from the roots, and may have an effect on the germination of the alkaloid-containing seeds or the seeds of other plants.

The presence of alkaloids in the seeds may protect them from birds. This does not seem to have been suggested before, and is a variant of the antiherbivore hypothesis. One objection to this idea is that the toxicity of many alkaloids is highly selective; however, even the non-toxic alkaloids may well have an exceedingly unpleasant taste, which would act as a conditioning stimulus rather than as a toxic factor, and may thus be a more effective method of protection. So far as poisoning is concerned, rabbits and many insects (for example) are immune to the alkaloids of Atropa belladonna (25), a few grams of the leaves of which are lethal to man. Nevertheless, as mentioned above, a selective advantage need only be slight in order for the trait conferring it to become widespread in relatively few generations, so those workers who have reported that alkaloids give no appreciable protection against herbivores may merely have failed to detect a slight but definite effect. Besides, the fact that rabbits are immune has little bearing on any protective effects alkaloids may have against other species of animals.

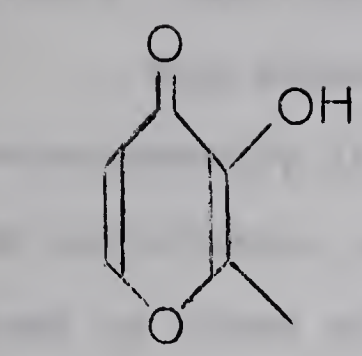
In this connection it may be apposite to mention that there are other secondary plant products that are not known to have a function and any distinction between alkaloids and these substances

on purely chemical grounds may have no biological validity. Examples of these are maltol (from larch bark); yangonin (from kava shrub root); umbelliferone (from spurge laurel bark); khellin (from Amni visnaga); and santonin (from various Artemisia species). The structures of these substances are shown in Figure 2.

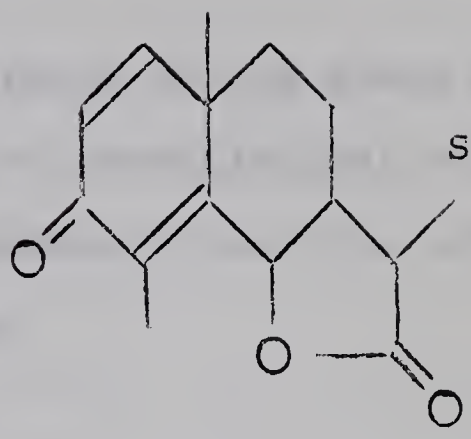
It is easy to see that if these compounds contained nitrogen they would undoubtedly be classified as alkaloids because of their close structural similarity to the types of compound usually given that name; all that would be required is the substitution of a nitrogen atom for an oxygen. This suggests that any distinction on a biological rather than a chemical basis between alkaloids and substances such as the above may be a highly artificial one. In properties other than those based directly on the presence of nitrogen the oxygen compounds resemble the group of alkaloids as a whole - organic heterocyclic structures, display of physiological effects on animals, and so forth.

The investigation of alkaloid function, therefore, may possibly be validly regarded as part of a larger problem - that of the function of limited-distribution plant products as a whole.

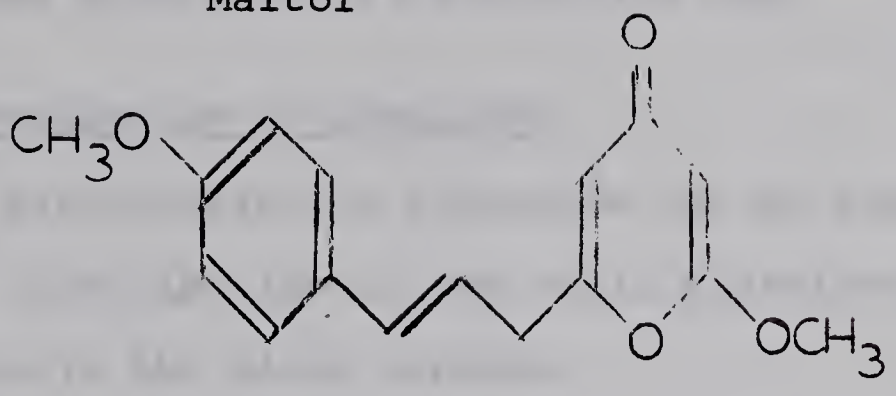
In this larger perspective we can see that the range of plants containing these products is greatly increased through the addition of the non-nitrogen-containing varieties, although the distribution of individual substances or related groups of substances is just as limited as before. The only major difference in overall distribution patterns is that the alkaloids are commoner in the



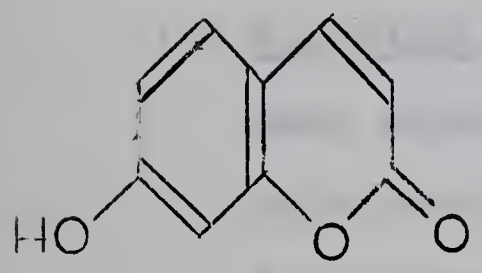
Maltol



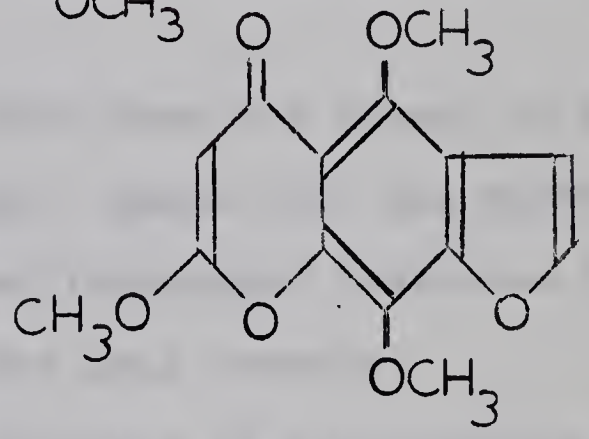
Santonin



Yangonin

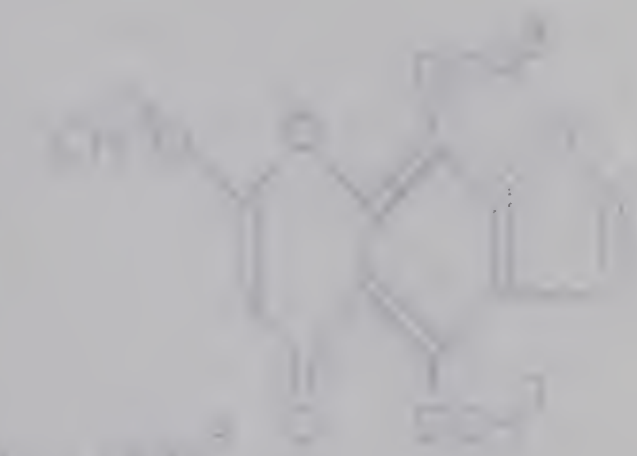


Umbelliferone



Khellin

Figure 2. Structures of some naturally-occurring alkaloid-like O-heterocycles.



higher vascular plants and the nitrogen-free substances in the lower plants, particularly the fungi.

The above facts, combined with the facts of the great chemical heterogeneity involved and the commonness of physiological effects on herbivores, are consistent with the hypothesis that the role of most of these substances is a protective one.

4. The distribution of alkaloids

The distribution of alkaloids can be considered from three viewpoints: distribution in the cell; distribution in the plant; and distribution in the plant kingdom.

(a) Distribution in the cell. This does not appear to have been extensively investigated. James (25) and McKee (45) both mention briefly that the literature indicates that alkaloids are localized in the cell vacuole.

(b) Distribution in the plant. Patterns of distribution of alkaloids in the plant differ widely, but James (25) reports that the commonest pattern is one in which the alkaloids accumulate most rapidly in growing tissues. This seems to be independent of the site of synthesis. It is, however, conceivable that accumulation in growing tissues may be merely a mass action effect. For example, tobacco alkaloids are synthesized - apparently exclusively - in the root, and are translocated to the leaves. On the other hand the lupine alkaloids seem to be synthesized exclusively in the green parts (10, 28), where they remain, except for such quantities as reach the seeds. Mention may also be be made of the cinchona alkaloids, which occur mainly in the bark of the tree (25).

(c) Distribution in the plant kingdom. It was once believed that alkaloids were of extremely limited distribution, but extensive surveys using modern analytical methods, as reported by Mothes and Romeike (50), have revealed that a great many species of plants contain alkaloids.

In 1924, Henry (23) could write: "Alkaloids have been found in comparatively few natural orders (sic) of plants: the Ranunculaceae, Papaveraceae, Fumariaceae, Solanaceae, Leguminoseae and Apocynaceae and typically rich, and the Rosaceae, Graminaceae and Labiateae typically poor in these constituents, while the Compositae occupy an intermediate position."

A more up-to-date outline of the distribution of alkaloids is given in Table I.

5. Isolation and analysis

Alkaloids vary so much in their physical and chemical properties that few generalizations can be made about methods of handling them, and Cromwell (14) discusses such as can be made.

Isolation usually involves air-drying of the plant tissue, followed by extraction with an organic solvent and various processes for removing impurities. Certain alkaloids are better isolated by extraction with aqueous solvents, followed by a series of pH adjustments and solvent partitions. Often, however, specific alkaloids may require special treatment, usually because of instability or unusual solubility properties.

Table I. Alkaloid-bearing classes, orders, and families

Class or Subclass	Orders	Families
Dicotyledones (subclass)	Anonales	Anonaceae
	Aristocholiales	Aristocholiaceae
	Asterales	Compositae
	Berberidales	Berberidaceae, Menispermaceae
	Boraginales	Boraginaceae
	Cactales	Cactaceae
	Campanales	Lobeliaceae
	Chenopodiales	Aizoaceae, Chenopodiaceae
	Contortae	Apocynaceae, Asclepiadaceae
	Cucurbitales	Caricaceae
	Garryales	Garryaceae
	Geraniales	Rutaceae
	Hamamelidales	Buxaceae
	Laurales	Lauraceae, Monimiaceae
	Leguminosae	Papilionaceae
	Loganiales	Loganiaceae
	Lythrales	Puniciaceae
	Magnoliales	Magnoliaceae
	Malpighiales	Erythroxylaceae
	Piperales	Piperaceae
	Ranales	Hernandiaceae, Nymphaceae, Ranunculaceae
	Rhamnales	Rhamnaceae
	Rhoadales	Papaveraceae

Table 1. (Continued)

Class or Subclass	Orders	Families
Dicotyledones (continued)	Rosales	Calycanthaceae
	Rubiales	Campanulaceae, Rubiaceae
	Rutales	Rutaceae
	Solanales	Convolvulaceae, Solanaceae
	Umbelliflorae	Umbelliferae
Monocotyledones (subclass)	Amaryllidales	Amarillidaceae
	Dioscoriales	Dioscoriaceae, Roxburghiaceae
	Graminales	Graminaceae
	Liliales	Liliaceae, Steminaceae
	Orchidales	Orchidaceae
	Palmales	Palmaceae
Gymnospermae	Coniferae	Taxaceae
	Gnetales	Gnetaceae
Pteridophytae	Equisetales	Equisetaceae
	Lycopodiales	Lycopodiaceae
Ascomycetes	Erysiphales	Hypocreaceae
Schizomycetes ..(phylum)	Actinomycetales	Streptomyceaceae

This Table is compiled from data contained in References 1, 14, 40.

A further difficulty is that isolation methods described as quantitative are frequently published with no assurance that complete extraction has in fact been achieved (16, 32, 39, 44). The fact that an extraction is exhaustive with a given solvent is no guarantee that another solvent might not remove substantially greater amounts of alkaloid (21). For preparative purposes this is of little consequence, but if quantitative analyses for alkaloid are to be performed on plant tissues, then completeness of extraction is clearly of paramount importance.

Analytical methods employed to 1955 also are summarized by Cromwell (14), and few significant developments have been made since then. Methods of estimation usually involve precipitation with one of the many reagents that have been described for this purpose, followed by analysis of the precipitate. Direct titration for isolated alkaloid can also be used, and Cromwell cites the use of polarographic and spectrophotometric methods, both of which are much more sensitive than the other methods named above.

Separation methods used in the past have been the traditional organic chemical methods - fractional crystallization and distillation etc., but in recent years every known type of chromatography has been successfully applied to alkaloid separations (19, 85). Along with the improvements in separation techniques are coming advances in microanalytical methods, but except in the cases of the alkaloids with the greatest commercial importance, such methods have not yet become widespread.

6. Metabolism

In recent years a flood of publications has appeared describing work on the biosynthesis and metabolic interconversions of alkaloids, but the accumulation of factual information still lags far behind the wealth of hypotheses that have been put forward. The most influential of these have been by Robinson (62), and more recently Marion (42), Leete (35) and Wenkert (88) have added to and modified Robinson's ideas, as has Robinson himself (61).

The chief feature of Robinson's work is that he pointed out the close structural relationships between alkaloids and other natural products, particularly amino acids and isoprene units.

Many chemical syntheses, using Robinson's postulated biosynthetic precursors, have been carried out, and when performed under conditions of moderate pH and temperature they have all too frequently been referred to as syntheses under physiological or even biological conditions - or as physiological syntheses (see, for example, references 24, 64, 65, 66). This is, to say the very least, misleading; there is no justification whatsoever for calling such conditions biological. Biological conditions involve enzymes, cofactors, membranes, highly-organized microstructures, structure-bound catalytic systems, strictly-localized concentration-, composition-, and pH-gradients, as well as specifically biological, "activated" forms of many molecules. To date, the only place where true biological conditions are found is in biological systems. Robinson's own warning (61) has only too often been forgotten:

"It is fully recognized that laboratory analogies may be fallacious. In fact the biologist can justifiably complain that the organic chemist is not satisfied until he has made biological reactions 'go' without enzymes or co-enzymes.

"It is an achievement but not necessarily a contribution to plant physiology."

The experiments that have the most direct significance in biosynthetic studies on alkaloids are those designed to trace metabolic pathways in the tissues that actually form these alkaloids in the so-called "natural" state.

There are further fallacies that have been inherent in many investigations of alkaloid metabolism, these being listed and discussed by Dawson (15). Chief among these is what he calls "failure to recognize the importance of determining the sites of syntheses of the alkaloid in the plant body as opposed to sites of accumulation." He cites (in 1948) sixteen investigations - including two of his own - of alkaloid syntheses in which the tissues employed were actually incapable of forming the alkaloid in question. Since his warning was published this error has become much less common.

It appears, therefore, that there has been an inordinate amount of careless and inexcusably loose thinking with regard to investigations of the metabolic relations of alkaloids. Nevertheless much valuable work has been done, particularly on the alkaloids of the greatest commercial importance - the opium and tobacco alkaloids (See, for example, 3, 22, 34, 46, 51, 67, 82). A number of others

with particularly obvious structural resemblances (Figure 1) to amino acids and other well-known metabolites have also been investigated, for instance harman (structurally related to tryptophan), and reserpine (related to tryptophan and to shikimic acid).

A recent review (57) summarizes and integrates the work done on alkaloid metabolism up to June 1963. Speaking generally, it appears that Robinson's views have been vindicated, and that almost all alkaloids so far investigated are in fact derived from one or more of a fairly small number of amino acids. Furthermore, notwithstanding the above warning about loose use of the term 'physiological conditions', certain of the initial cyclizations may in fact be nonenzymic, although why one type of cyclization to form a certain kind of alkaloid skeleton should occur in one plant species, and another type of cyclization in another species, and in a third species no cyclization at all, is not clear. Once the cyclization has taken place, further modification seems to be enzyme-catalyzed.

Ib. INTRODUCTION TO THE LUPINE ALKALOIDS

1. Reason for choice

Research into the physiology of alkaloids has only recently become common, and before any general theory can be given widespread experimental support, many aspects of the metabolism of individual groups of alkaloids must be investigated.

The present study concerns a single such group, and was undertaken with these facts in mind. At this early stage in the study of alkaloids there seems to be little to choose among different types of alkaloids from the point of view of significance; as regards our knowledge of the physiology of alkaloids generally, all are very much alike - that is, not much is known about any of them - so the choice of a group to work with must either be made on the basis of convenience or be completely arbitrary. The reasons for choosing the lupine alkaloids for this study are listed below:

(a) Their chemical structures are sufficiently complex to justify their being considered "true" alkaloids, but they are not so complex or unstable that they are difficult to work with.

(b) There exist a fairly large number of different lupine alkaloids with several variations on the basic carbon-nitrogen skeleton, so that metabolic interconversion studies are likely to be rewarding.

(c) They occur in a wide variety of species and at least twenty-five genera spread over four families - the Leguminosae

(particularly in the subfamily Papilionaceae), Chenopodiaceae, Berberidaceae, and Papaveraceae (36), so they are likely to be of more general significance than most groups of alkaloids.

Those genera, other than Lupinus itself, that have been shown to include species containing quinolizidine alkaloids, are listed below in alphabetical order:

Adenocarpus	Cytisus	Retama
Ammodendron	Equisetum	Solanum
Ammothamnus	Genista	Sophora
Anabasis	Hovea	Spartium
Anagyris	Laburnum	Templetonia
Baptisia	Leontice	Thermopsis
Calycotome	Piptanthus	Ulex
Chelidonium	Podalyria	Virgilia

(d) One of their chief sources, Lupinus luteus var. Romulus, has the advantages of being a readily available and easily grown annual with tissues that are easy to handle; for these reasons this species was chosen for this study.

2. History

The alkaloids of yellow lupine seeds were first isolated by Cassola in 1835. The first of these alkaloids to be isolated in a pure state - but from broom, not lupine - was sparteine (Stenhouse, 1852); its isolation from lupine seeds was not achieved until 1904, by Willstätter and Marx. Lupinine was first purified by Baumert in 1881, and new lupine alkaloids have since then been discovered at

frequent intervals until now over sixty are known. For details of this history, and the original sources of this work, see Leonard (36).

3. Distribution and chemistry

Details of the distribution and chemistry of the lupine alkaloids can be found in Leonard (36, 37). Those of the alkaloids that have been characterized and that occur in Lupinus species are listed in Table II together with their molecular formulae and their sources.

The chemical structure common to them all is the quinolizidine ring, (Figure 3), which was in fact unknown until discovered in the lupine alkaloids. Many members of this group of alkaloids actually contain two fused quinolizidine ring structures, and the various carbon-nitrogen skeleta that have been characterized to date are clearly seen in Figure 3. Details of the elucidation of the structures, and of their stereochemistry, can be found in Leonard (36, 37).

4. Metabolism

In recent years, much valuable work has been done on the biosynthesis of lupine and other alkaloids. A useful review, summarizing the work reported to June 1963, has been prepared by Ramstad and Agurell (57). They conclude, from general observations of the literature on the subject, that the N-heterocyclic rings of many, and possibly all, alkaloids are formed by non-enzymic Mannich cyclizations, and that only the later reactions in the biosynthetic routes are enzyme-catalyzed.

Table II. Alkaloids found in Lupinus species

Alkaloid	Formula	Lupinus species in which found
Anagyrine	$C_{15}H_{20}N_2O$	L. caudatus, laxiflorus, macounii, pusillus.
Angustifoline	$C_{14}H_{22}N_2O$	L. angustifolius, perennis.
Epilupinine	$C_{10}H_{19}NO$	L. pilosus, digitatus.
Homothermopsine	$C_{17}H_{24}N_2O$	L. lanceolata.
Hydroxylupanine	$C_{15}H_{24}N_2O_2$	L. albus, angustifolius, hiliaranus, perennis, polyphyllus, sericeus, wyethi.
Isolupanine	$C_{15}H_{24}N_2O$	L. caudatus, sericeus.
Isosparteine	$C_{15}H_{26}N_2$	L. caudatus, pusillus, sericeus.
Lupanine	$C_{15}H_{24}N_2O$	L. albus, angustifolius, arboreus, caudatus, kingii, laxus, perennis, polyphyllus, sericeus, macounii, pusillus, arboreus, hartwegi, mutabilis, pilosus, wyethi.
Lupanoline	$C_{15}H_{24}N_2O_2$	L. sericeus.
Lupilaxine	$C_{15}H_{24}N_2O_2$	L. sericeus, laxus.
Lupinine	$C_{10}H_{19}NO$	L. luteus, niger, palmeri.
Pentalupine	$C_{16}H_{30}N_2O$	L. palmeri.
Sparteine	$C_{15}H_{26}N_2$	L. pusillus, arboreus, barbiger, caudatus, laxus, luteus, mutabilis, niger, sericeus, digitatus, wyethi.
Thermopsine	$C_{15}H_{20}N_2O$	L. caudatus, corymbosus.

Condensed from James (17).

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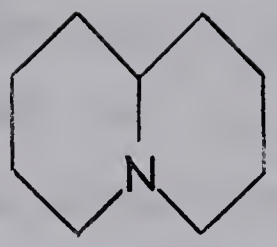
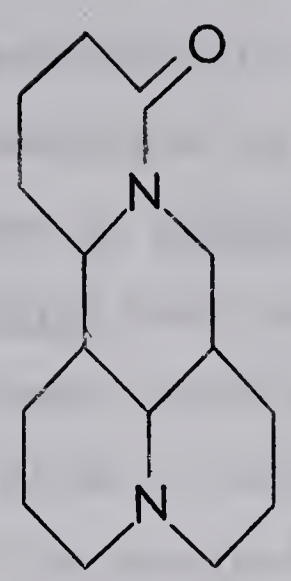
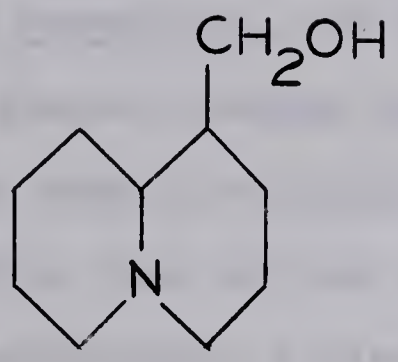
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]



Quinolizidine

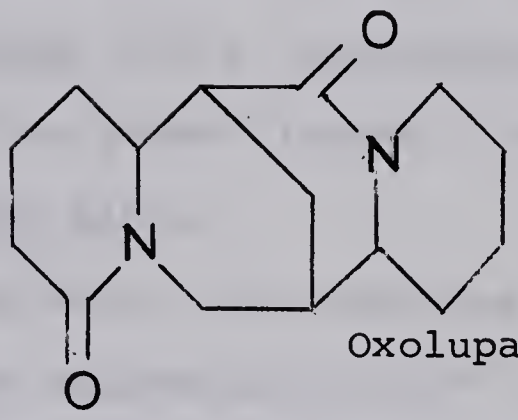


Matrine

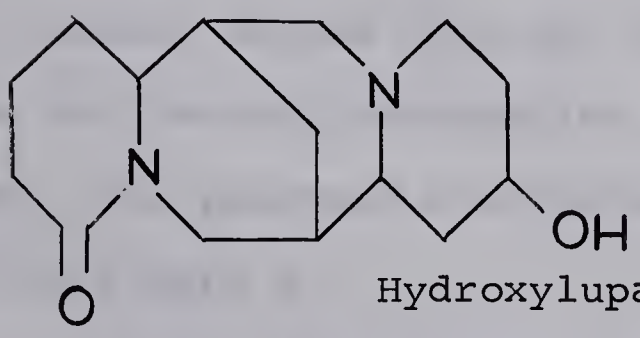


Lupinine

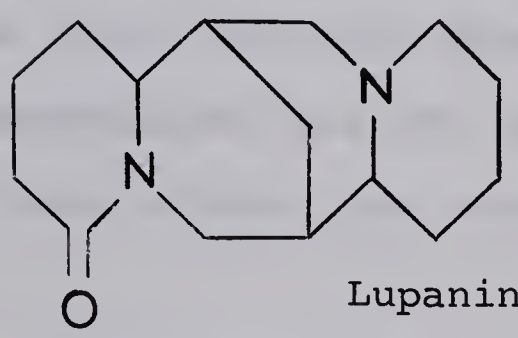
Sparteine



Oxolupanine



Hydroxylupanine



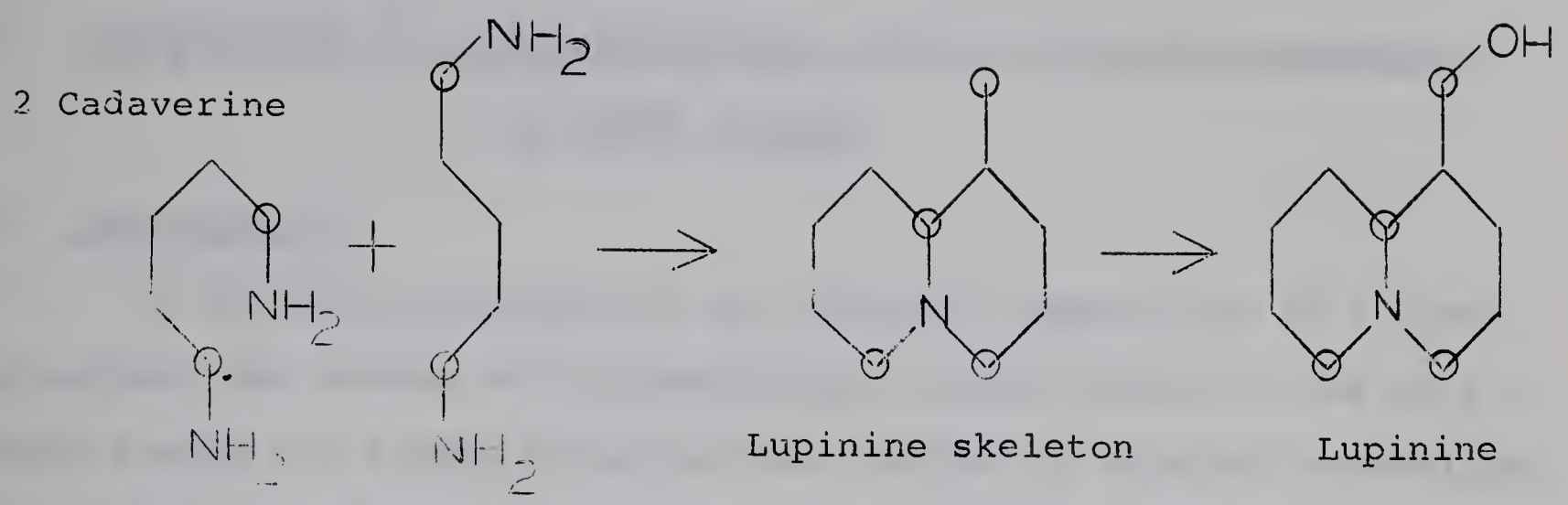
Lupanine

Figure 3. The structures of quinolizidine and some quinolizidine alkaloids.

With particular reference to the quinolizidine alkaloids, the work of Schütte and his coworkers has proved particularly valuable (68, 69, 73, 75, 76, 77, 79, 81, 86). Schütte's technique is usually to allow 9-10 week old shoots of L. luteus (although L. angustifolius, L. albus, Sarothamnus scoparius, and Chelidonium majus were used in some experiments) to absorb labelled precursors through curtailed roots, then to analyze them for labelled alkaloid after a two-day incubation period. This long period of incubation is required because of the slow rate of metabolism of alkaloids. (It should be noted here that grafting experiments by Kazimierski and Nowacki (28) have shown that in L. albus, L. pilosus, L. mutabilis, and L. angustifolius, the alkaloids are synthesized exclusively in the green tissues. Presumably this holds for other species of Lupinus also.)

Up to the time of the review mentioned above, two pathways were considered possible from lysine-2-C^{14} and cadaverine-1,5-C^{14} to lupinine, these having been described in the review, but later work (70, 78) involving systematic degradation of the labelled alkaloids thus formed, showed that one of these pathways (that involving piperidine and tetrahydroanabasine as possible intermediates) was not operative. The pathways elucidated to date from these studies are summarized in Figure 4.

It should be noted that it has not been found possible to reverse any of these reactions in vivo. The conversion of lupanine to hydroxylupanine, and its nonconversion back to sparteine have been confirmed by Birecka and Sebyła (9) in L. albus by observing the products formed from C^{14}-labelled lupanine.



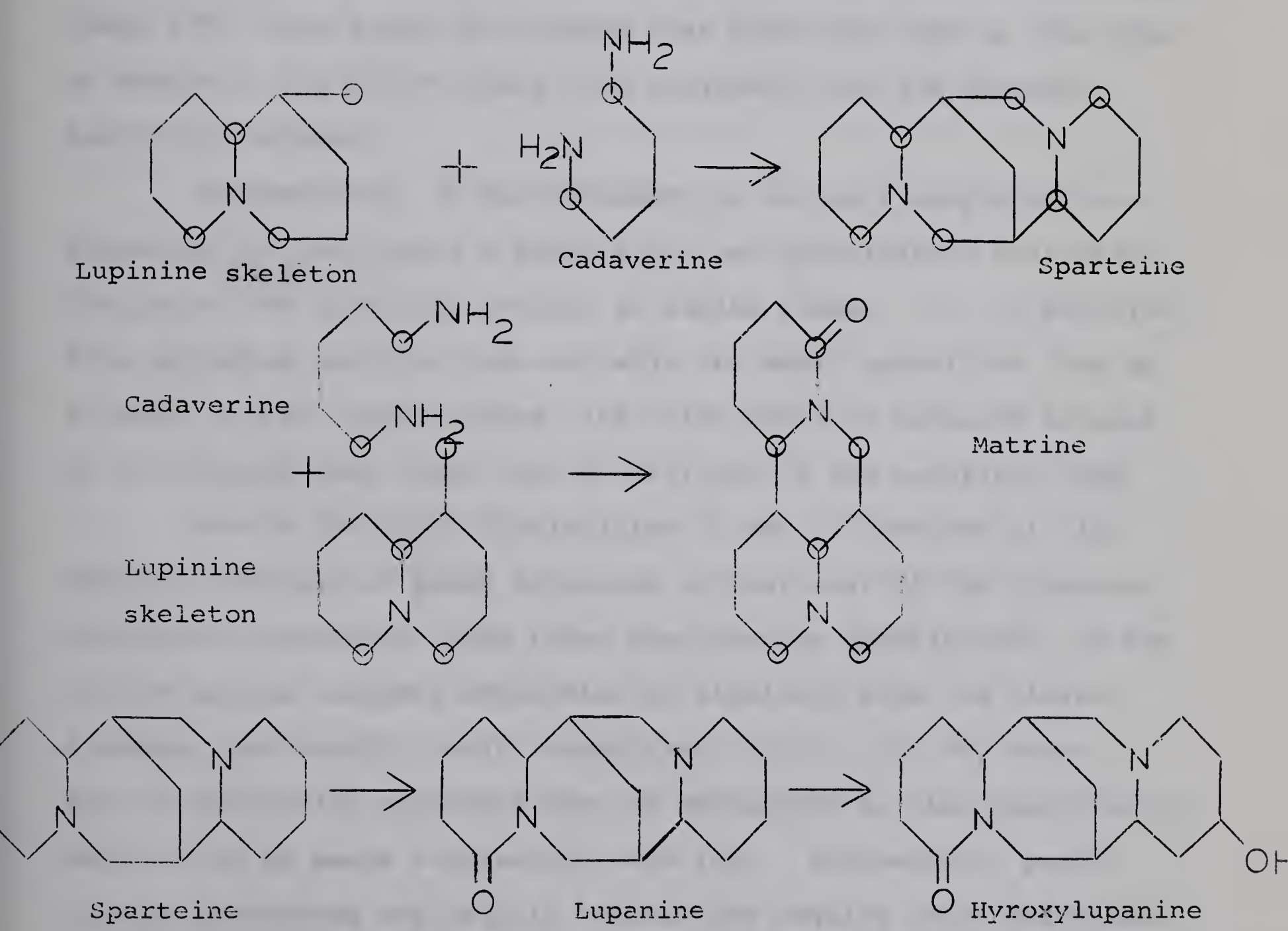
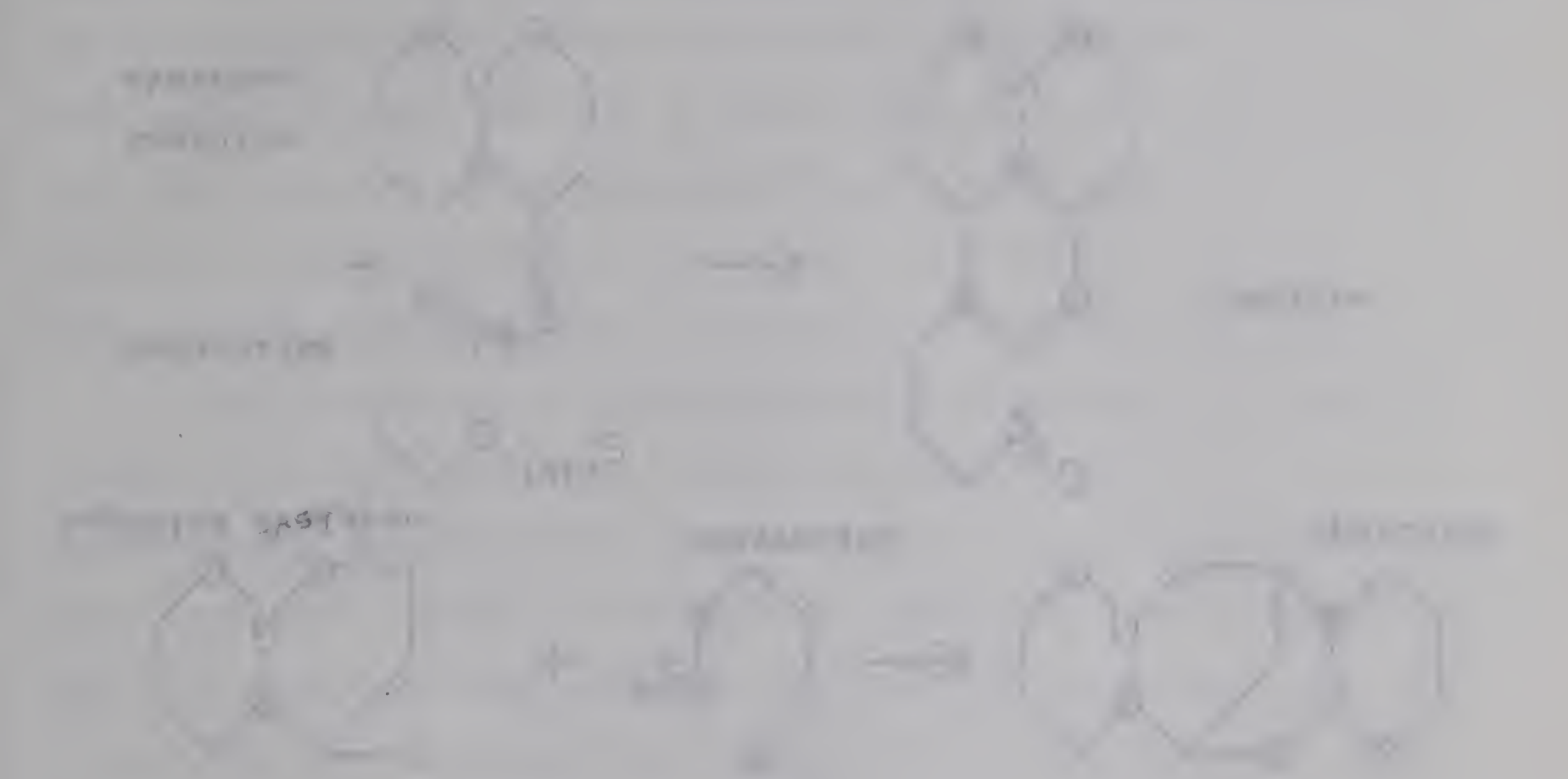


Figure 4. Metabolic interrelationships of the lupine alkaloids. Circles indicate C^{14}-labelled metabolites. In the product alkaloid, the label is distributed uniformly among those carbons that are labelled at all.



II. DEVELOPMENT OF A MICROANALYTICAL METHOD FOR LUPINE ALKALOIDS IN PLANT TISSUES

1. Introduction

A detailed knowledge of the alkaloid composition of a plant throughout the course of its development should provide clues as to which stages are likely to be the most active in alkaloid metabolism. James (25) lists those few studies that have been done on this type of analysis, but all of these have employed crude and obsolete analytical methods.

Consequently, it was necessary to devise a completely new procedure for performing a qualitative and quantitative characterization of the alkaloids present in lupine tissue. If, in addition, this procedure could be made suitable for small quantities (say up to about 5 g) of lupine tissue, its value would be enhanced because of the economy that could then be realized in the materials used.

One of the chief difficulties in the old methods of biochemical analysis of plant alkaloids is that most of the classical extraction procedures, even those described as quantitative, do not in fact ensure complete extraction of alkaloids from the tissue - a common, and usually tacit, assumption (16, 32, 39, 44) being that an extraction procedure that is exhaustive is also quantitative, which is by no means necessarily true (21). Furthermore, purification procedures are usually tedious and require large quantities of materials; transfer losses occur readily; and conventional analytical methods are often difficult to use with minute quantities of alkaloid (14).

In addition, solvent partition methods, when used alone, are unsatisfactory for the quantitative isolation of alkaloids from plant tissues because they tend to be tedious, and involve large transfer losses as well as difficulties due to the formation of troublesome emulsions.

Some attempts, notably by Lee (32), Mattocks (44), and Tompsett (85) have been made to use ion-exchange resins for isolation of alkaloids from the tissues in which they occur, and to a lesser extent for the separation of alkaloids from each other, but these methods are of limited usefulness, and in particular, none is suitable for lupine alkaloids. Lee's method, originally designed for the fractionation of opium, necessitates beginning with fairly large quantities (several grams) of alkaloid that has already been isolated in crude form; Mattocks' procedure is useful only for very large quantities; Tompsett's extraction procedure is so vigorous as to destroy lupine alkaloids, and all three employ Dowex 50, which we have found to bind lupine alkaloids irreversibly. Acid cannot be used for elution of lupine alkaloids from this resin, even at high concentrations, because the resin absorbs these alkaloids from such solutions; basic eluants are unsuitable since several of the alkaloids are unstable, or insoluble, or both, in the unionized form. The method finally developed is described below.

2. Experimental

Apparatus. A Beckman Model B Spectrophotometer with matched 1-cm Pyrocell quartz cells was used for the nephelometric determinations.

Reagents. Duolite C-63 phosphonic acid cation exchange resin (from the Chemical Process Co., Redwood City, Calif., or, under the name Bio-Rex 63, from the California Corp. for Biochemical Research, Los Angeles) was washed continuously in a column with twice the volume of 6 N HCl required to discharge the yellow color, followed by demineralized water until the effluent gives no reaction with silver nitrate solution. The resin was removed from the column, allowed to dry in air (this does not damage the resin), and dry-screened to 20- to 40-mesh.

Two solvent systems were used for paper chromatography; the first was the hydrochloric acid-butanol-toluene system described by Reifer et al. (59); the second was glacial acetic acid-tertiary amyl alcohol-water (1:4:7), the lower phase of which was used for equilibration and the upper for development. These are abbreviated to HCl Bu-Tol and t-Am HOAc respectively, in the remainder of the thesis.

The reagent for nephelometric microdetermination of the alkaloids, based on the method of Reifer and Niziolek (58), was prepared as follows; 23.2 g of KI and 19.2 g of KBr were dissolved in about 100 ml of water; 22.0 g of crystalline I_2 was now added with continuous stirring and the total volume made up to 240 ml with water then to 400 ml with 1 N NCl. A slight precipitate remained and the supernatant was decanted off and stored in the refrigerator until required. This method of preparing the reagent was faster than that described by the originators and avoided the use of elemental bromine.

In some early experiments the chromatograms were sprayed with 1% I_2 in $CHCl_3$ to reveal the alkaloid spots without changing them chemically; this spray forms a transient yellow or brown stain with many alkaloids and other substances. This stain was later found to be unreliable and in later experiments one of the following stable modications of Dragendorff reagent was used: Dragendorff-Palmer reagent (55) or Dragendorff-Munier reagent (53). Both of these contain potassium iodobismuthate as the active ingredient and both reveal the alkaloids as intense orange or pink spots against a yellow background.

Alkaloids were obtained from Mann Research Laboratories, Inc., New York, N.Y. All other reagents named, including the components of the phosphate buffers and chromatography solvents, were analytical grade and obtained from Fisher Scientific Co., Ltd., Edmonton, Alta., or Montreal, P.Q.

Procedure. About three grams of fresh or frozen tissue was ground to a uniform consistency with enough water to bring the total volume to 5 ml, in a 12-ml heavy-duty graduated clinical centrifuge tube, using a glass rod as a pestle. Lupine tissue is so friable that very thorough disintegration was readily achieved by this means. The residue was resuspended in the same volume of water as was the original tissue and the process repeated for a total of four extractions, this number having been found sufficient to remove all detectable alkaloid. The final residue was discarded and the aqueous extract mixed with its own volume of 0.2 M potassium phosphate buffer, pH 6.50. If necessary, the pH was adjusted to exactly 6.50 with the appropriate 0.1 M buffer component.

About 2.5 g (dry weight) or resin which had been equilibrated with 0.1 M potassium phosphate buffer, pH 6.50, was placed in a 1-cm diameter glass column. The buffered extract, centrifuged to remove debris that may have precipitated on standing, was then passed through the column at a flow rate not exceeding 1 ml per minute. The effluent, which was thus cleared of alkaloid, was discarded and the resin bed washed with about 25 ml of water to remove residual plant material.

In some early experiments the resin, instead of being contained in a column through which the extract was passed, was sealed in a short length of dialysis tubing together with a little of the equilibrating buffer. This sac was then left in contact with the extract for several hours with continuous stirring. This procedure was abandoned when it was found that the larger molecules (e.g. peptides) that the membrane was intended to exclude from the resin did not in fact interfere with the efficiency of the method, and that even after twenty-four hours contact the resin inside the dialysis tubing absorbed about 85% of the alkaloid, as opposed to the 99% absorbed in less than thirty minutes when the column was used.

The alkaloids were immediately eluted from the column with 50 ml of 6 N HCl (in which the resin and the alkaloid separately were found to be stable), also at a rate of 1 ml per minute. This flow rate was critical, since faster rates failed to elute all the alkaloid, and prolonged exposure of the alkaloid to the resin and acid together tended to destroy some of the alkaloid (although the resin alone did not cause this destruction). To

this acid eluate was added 1-2 ml of approximately 5 M $CaCl_2$, which precipitated any emulsifying agents that may have been carried along this far with the alkaloids. The eluate was chilled to just above its freezing-point and mixed with a slight excess of 10 N NaOH, also close to freezing; the chilling prevented the overheating that would otherwise have occurred on mixing, the maximum temperature reached being not more than 40° C, and usually less. A heavy precipitate of calcium salts formed at this stage and was removed by centrifugation.

The used resin was then discarded, since re-use sometimes gave rise to troublesome artefacts.

The basic solution was then extracted quickly with about ten three-ml aliquots of chloroform in a separatory funnel fitted with a Teflon stopcock (to avoid contamination of the extract with stopcock grease) and the extracts were added to 1 ml of glacial acetic acid (6), which is miscible with chloroform. This converted the alkaloids to the acetate forms, which are much more stable in chloroform than are the free alkaloids. The chloroform and acid were then evaporated off under reduced pressure or, in later experiments, in a stream of nitrogen to leave the alkaloid salts in the form of a white crystalline deposit, weighing a few milligrams. Haste was nevertheless necessary at this stage because prolonged (several hours) contact with chloroform can cause irreversible changes even in the ionized alkaloids (43). It was found that acetic acid did not cause partial decomposition of the alkaloids during evaporation to dryness, a difficulty encountered by Birecka et al. (6a) with hydrochloric acid.

The last traces of impurity were removed by washing the crystals in petroleum ether, in which the alkaloid salts are insoluble, dissolving them in 1 ml of 0.05 N HCl, and filtering or centrifuging the solution to remove insoluble material.

Aliquots of this solution (or the whole sample if so desired) were spotted on Whatman No. 3MM chromatography paper, and the chromatograms were equilibrated and developed in darkness at room temperature. Some of the alkaloids, although photosensitive, are not unduly so, and admission of light during the brief early stages of the extraction, prior to chromatography, had no detectable effect. If, on the other hand, light was admitted during the more prolonged period of time required for chromatography, considerable streaking and double spotting was liable to occur.

After development the papers were dried and stained. Since the various forms of Dragendorff reagent make the alkaloid unavailable for quantitative estimation the following technique was used to locate the alkaloids for elution from the paper: two identical chromatograms were run on each sample, and one of these was sprayed with the reagent. Using this stained chromatogram as a guide, the other one was marked for excision and elution of the spots for quantitative analysis. A margin for error was allowed in cutting out the spots, and as a check the remainder of the paper was sprayed with Dragendorff reagent. No detectable alkaloid ever remained behind. The alkaloid spots were excised and cut into narrow strips for elution with 1 N HCl. The volume of eluting solvent used was large enough to ensure complete elution, while keeping the alkaloid concentrated enough for estimation. If too small a volume was used there was

a danger of incomplete elution from the paper. Three milliliters of eluate, centrifuged to remove filter paper fibers that might interfere with the nephelometric estimation technique, was pipetted from each tube into a reaction vessel in an ice bath. If the concentration proved to be above the range most suitable for estimation, it was easy to dilute to a more favourable concentration range. Two milliliters of the modified Reifer reagent, which forms a colloidal complex with the alkaloid salts, was pipetted into each sample and into a 1 N HCl blank. After thirty minutes the samples were transferred to the spectrophotometer cells and the absorbance was measured at 820 mμ. This is the wavelength at which the iodine reagent was found to have maximum transmittance, and was chosen in preference to the Leitz filter B suggested by the originators of the photometric method, to yield maximum sensitivity for nephelometric measurements. Comparison with standard calibration curves yielded the concentration of alkaloid in each sample, from which the total quantity of alkaloid in the tissue could readily be calculated.

3. Results and Discussion

The efficiency of various commonly used extracting solvents was tested as follows: known quantities of alkaloids were mixed with plant tissue that did not already contain alkaloid, and re-extraction with the solvent in question was carried out, followed by quantitative estimation of the alkaloid extracted. In each

case, after extraction, the tissue was dried, the residue extracted with chloroform, and the chloroform extract tested qualitatively for alkaloid. The results are shown in Table III. The same solvents were tested, as was methanol, another commonly used solvent, on actual lupine samples, and in each case a chloroform extract of the dried residue contained alkaloid, except when water was the original solvent. This is contrary to the observation of Birecka and Nalborczyk (6), who reported equally efficient extractions with water and with methanol. The reason for the increased efficiency of water over acid solvents is unknown, but a possible explanation is that the acid solvents may have precipitated some tissue protein, trapping alkaloid in the coagulum. On the other hand, the cell contents are slightly acid to begin with, although of course not acid enough to cause coagulation; the alkaloids would therefore have been in the water-soluble salt form, so that they could be readily extracted with ordinary water. It may nevertheless be better to use a slightly acid buffer rather than water to make sure that the alkaloids <u>are</u> in the salt form in the case of tissues in which the pH is unusually high. Chloroform itself is unsuitable as an extracting solvent because some lupine alkaloids, when ionized, are insoluble in chloroform, and when unionized, are unstable.

The R_f values of the alkaloids in the two solvent systems used are shown in Table IV.

The calibration curves for the alkaloids are shown in Figure 5. The concentrations are expressed in molar units rather than in parts per million in order to render more meaningful the comparisons between absolute quantities of different alkaloids coexisting within the plant. It is clear that reporducibility is high with all alkaloids except oxolupanine, which is not a

Table III. Efficiency of extraction of alkaloids from plant tissues by different solvents

Solvent	Extraction efficiency	Alkaloid in $CHCl_3$ extract of residue
0.1 N HCl	78%	Present
0.1 M H_3PO_4	73%	Present
1.0 M H_3PO_4	37%	Present
0.1 M Phosphate buffer, pH 6.50	89%	Present
0.1 M KH_2PO_4	89%	Present
Water	100%	Absent

In each case 3 grams of tissue that did not already contain alkaloid, with 10 μ moles of added alkaloid, was extracted with four 5-ml aliquots of solvent.

Table IV. R_f values of lupine alkaloids in t-Am HOAc* and in HCl Bu-Tol** solvent systems

Alkaloid	R_f (t-Am HOAc)	R_f (HCl Bu-Tol)
Sparteine	0.06	0.26
Lupinine	0.30	0.37
Lupanine	0.52	0.32
Hydroxylupanine	0.18	0.21
Oxolupanine	0.86	0.79

Descending chromatography was performed at 27° C on Whatman #3MM paper.

R_f values vary by up to about ten per cent between runs.

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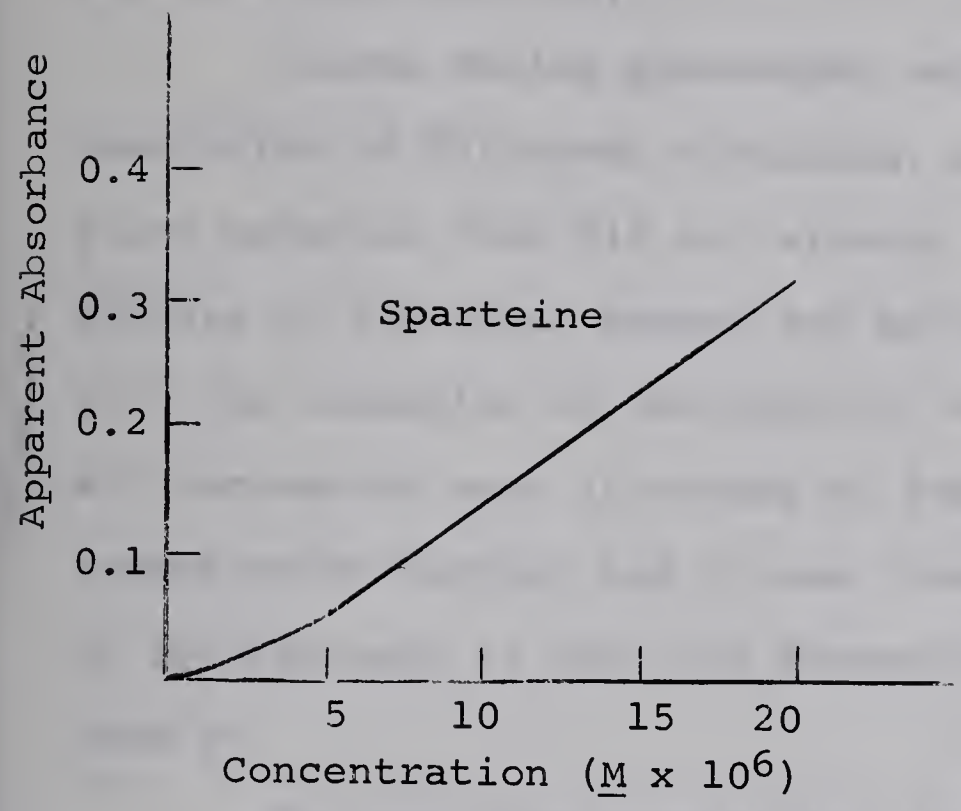
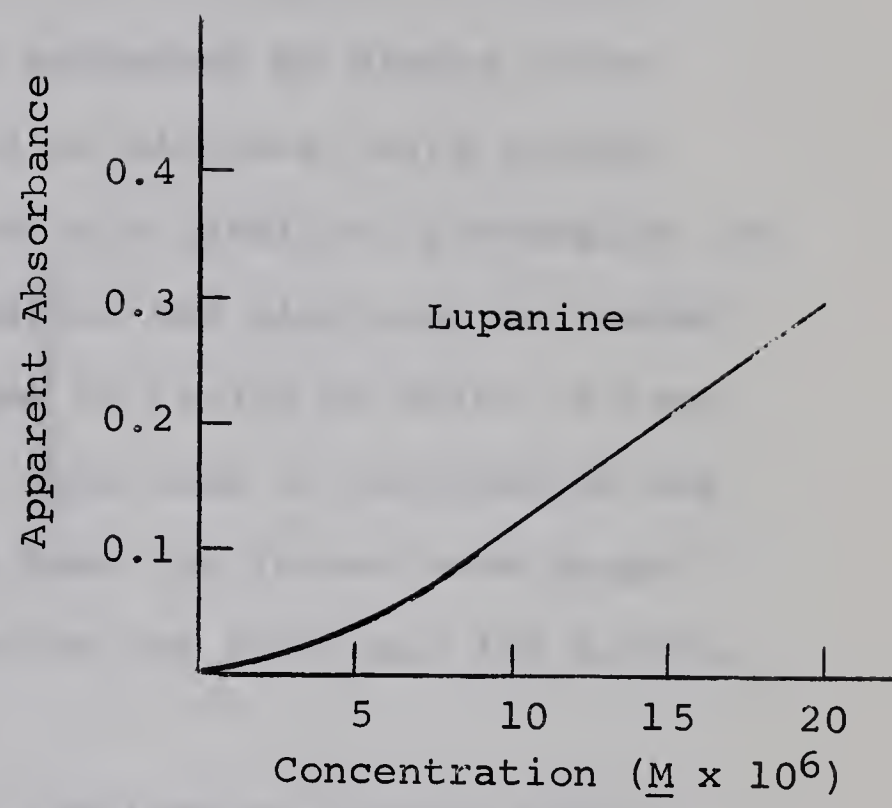
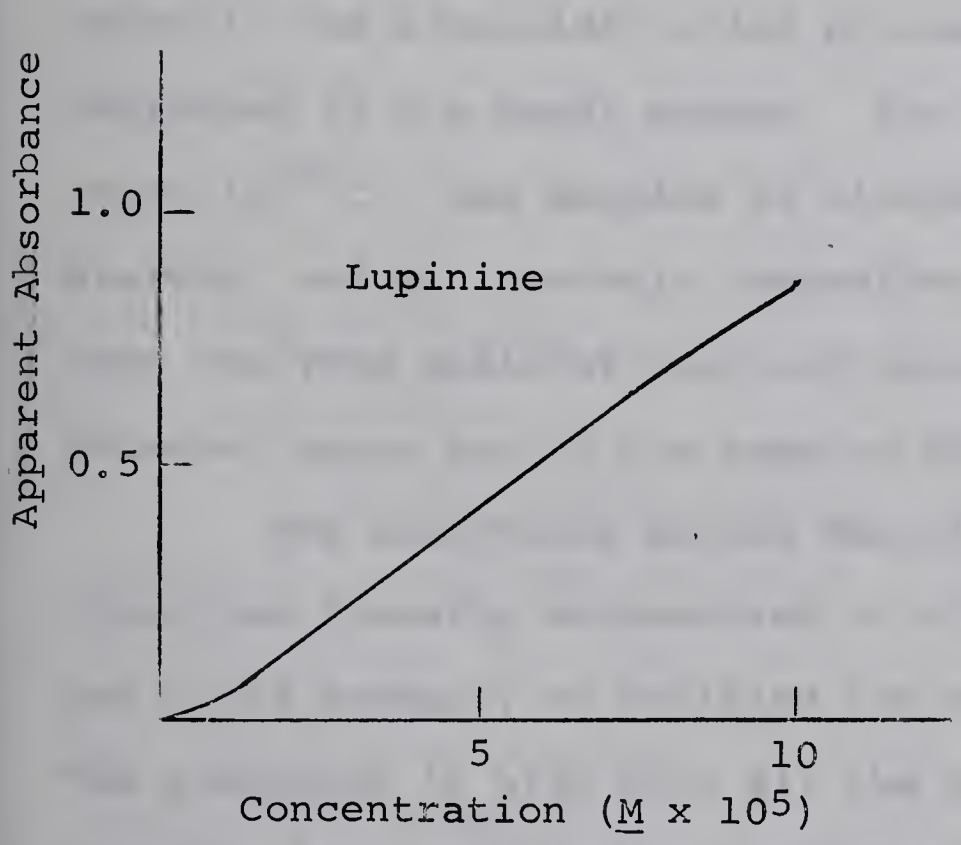
* t-Am HOAc: equilibrated with lower phase of tertiary amyl alcohol-acetic acid-water (1:4:7); developed with upper phase.

** HCl Bu-Tol: equilibrated with lower phase of HCl-butanol-water (3:4:9); developed with upper phase after addition of 1 volume toluene.







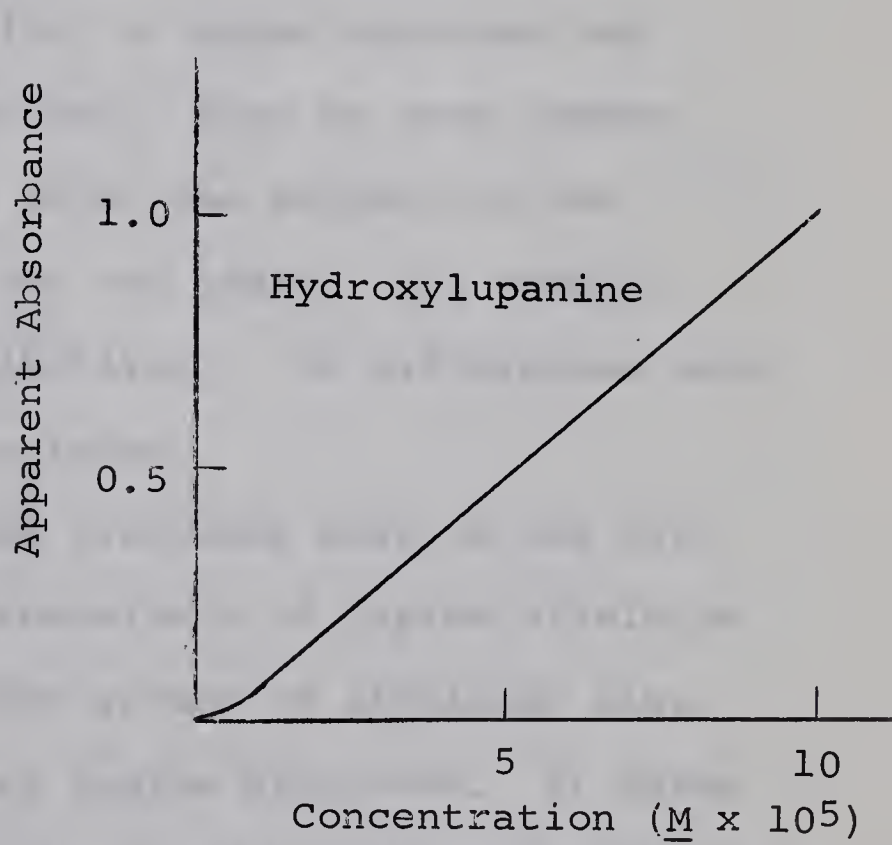
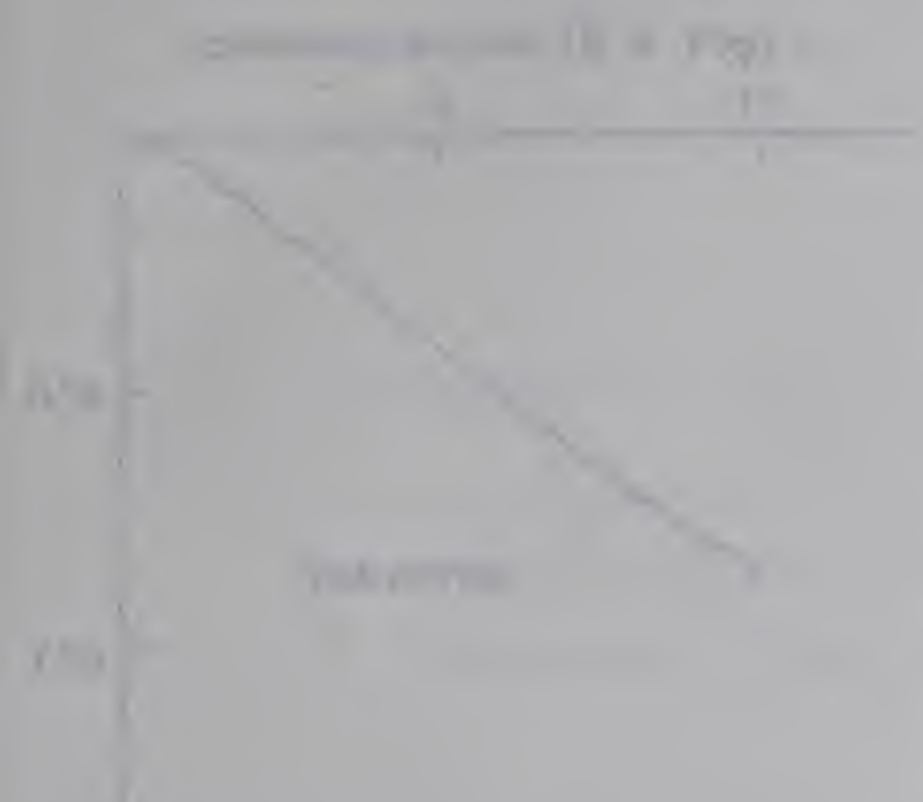
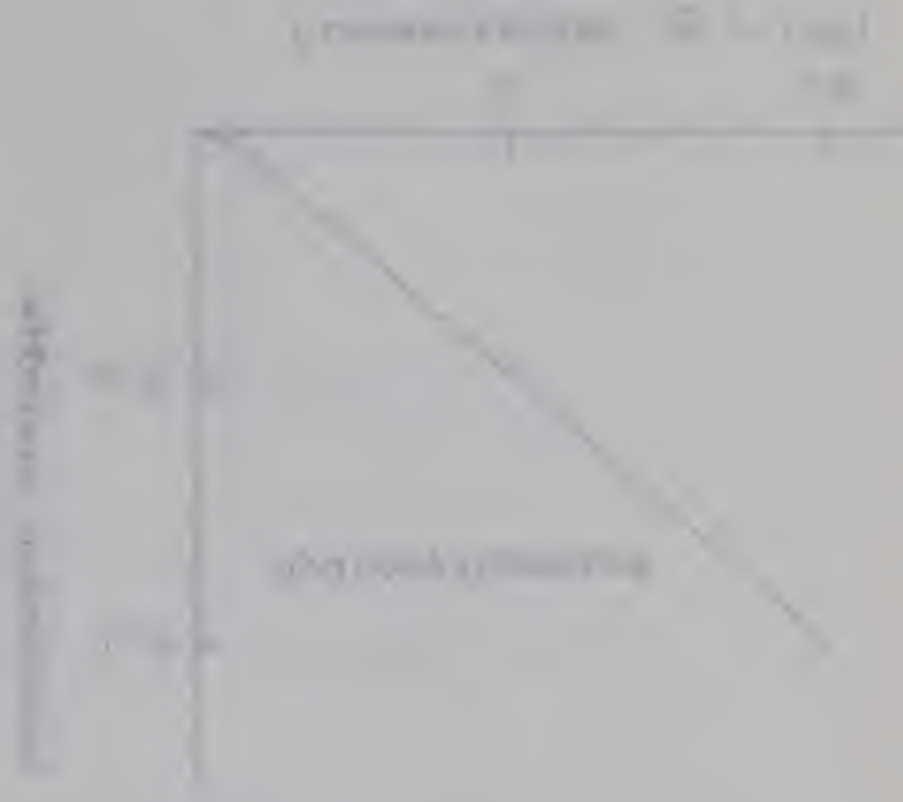
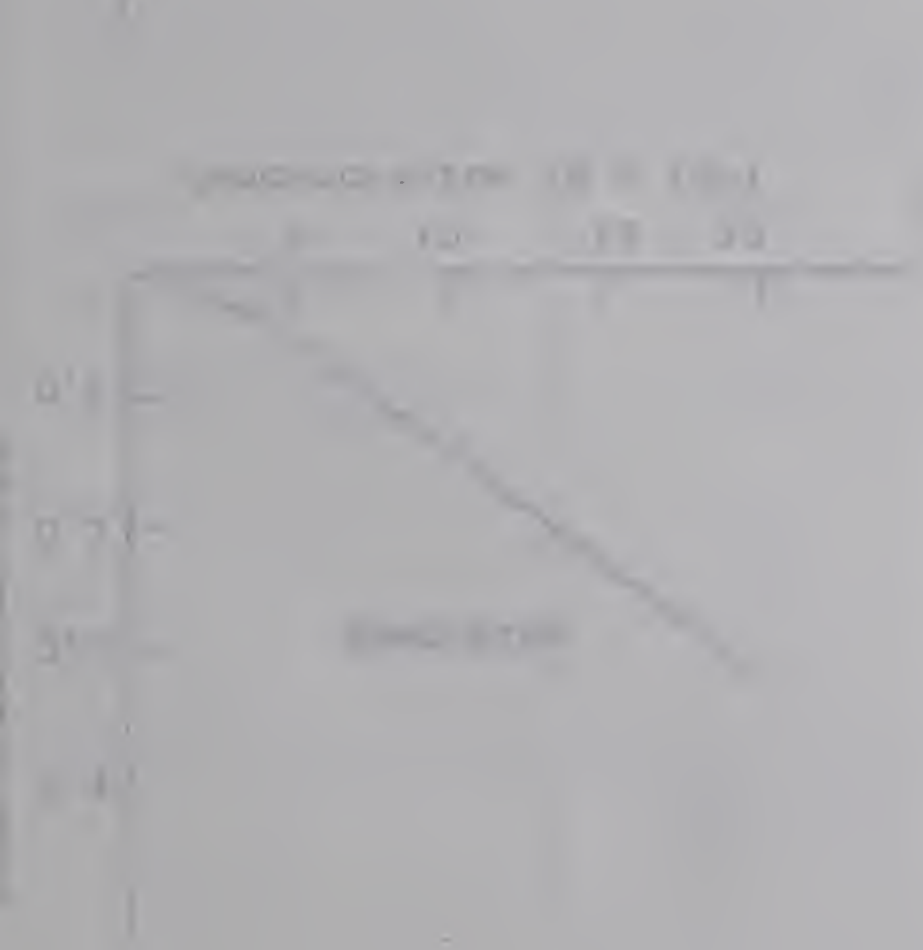
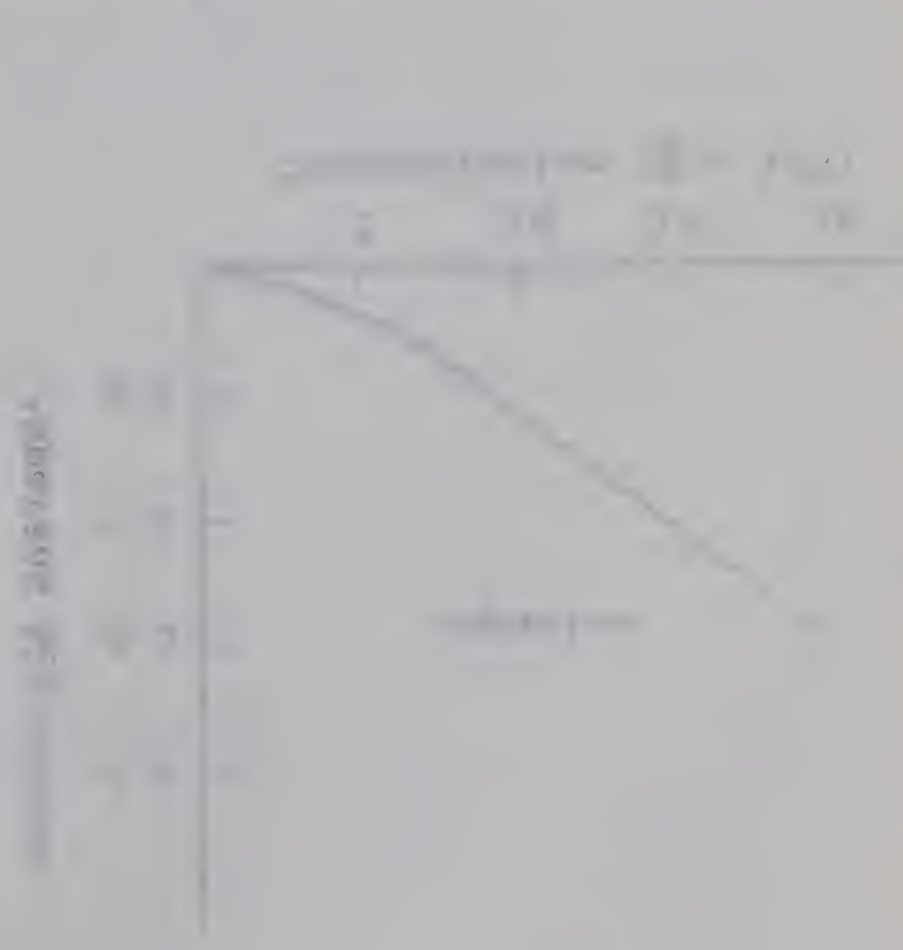


Figure 5. Calibration curves for four alkaloids with Reifer's reagent.









major alkaloid in L. luteus (63, 86, 87). It can nevertheless be detected qualitatively.

Losses during processing were estimated by mixing known quantities of different alkaloids, and of mixtures, with ground plant material that did not already contain alkaloid, processing the mixture in the usual manner and estimating the alkaloids recovered. With the exception of oxolupanine, some 55 to 75% of which is lost, all recoveries were in excess of 95%. The case of oxolupanine was investigated further and it was found that the losses were caused by inefficiency in both its adsorption on the resin and its elution from it.

To check the possibility that qualitative changes might occur in the alkaloids during processing, a lupine specimen was extracted in the usual manner. The extract, kept at room temperature (27° C), was sampled 15 minutes after the extraction was started, and at intervals thereafter for ten hours. All samples were analyzed qualitatively and quantitatively. No differences were detected among any of the samples thus taken.

The analytical method described overcomes most of the difficulties formerly encountered in microanalysis of lupine alkaloids and could probably be modified for other groups of alkaloids also. The precision is high with all the main lupine alkaloids. It makes possible both qualitative and quantitative serial analyses of plants at different stages of development. By this means it is possible to locate developmental stages at which alkaloid metabolism is most active, so that these stages can be concentrated on for closer study. It is also useful for the analysis of reaction mixtures of alkaloids in vitro.

III. STUDIES OF ALKALOID ONTOGENESIS IN L. LUTEUS VAR. ROMULUS

1. Introduction

Serial analyses of metabolites present in plants at successive stages of development should give at least a partial indication as to the metabolic relationships of these materials. For example, if one of a pair of closely related substances increases in quantity over a given period of time, and the other decreases at the same rate, this is an indication, although of course by no means a proof, that the second is being transformed into the first. If we were interested in such an interconversion, we would then know that the growth stage over which these changes took place would probably be a promising one to use in our investigation.

Some work of this nature has already been done with L. luteus (63, 86, 87), but this was done by use of obsolete and sometimes even crude analytical methods. For example, van der Kuy (86) used large numbers of plants, extracted and purified individual alkaloids and determined them by direct weighing. The consensus of these reports appears to be that lupinine is the major alkaloid in this species, and that at certain growth stages, particularly near the flowering stage, sparteine is also present in large quantities. Other alkaloids appear from time to time in smaller quantities.

All three of the above-named studies involved widely-spaced analyses over the entire life-span of the plant. Little if any

detailed information is available for the period during and immediately following germination, a period when the physiology of the plant is undergoing drastic changes. If the metabolism of the alkaloids is in fact associated with the physiology of the plant, changes in this metabolism might be expected to show up at this stage of the life-cycle of the plant. Furthermore, because of the rapid changes in alkaloid content that might be expected, or at least because of the rapid metabolism in general, they should be a good source of active material for *in vitro* studies. Another advantage is that they provide a tissue that can be readily obtained at short notice, and at relatively uniform stages of development.

2. Experimental

Apparatus. The amino acid analyses were carried out using a Beckman Model 120B Amino Acid Analyzer (Beckman Instruments Inc., Palo Alto, Calif.). A flash-evaporator by Buchler Instruments, Fort Lee, N.J., was used during preparation of the samples for analysis.

Seedlings were grown under uniform conditions in a small growth cabinet of the following design: the cabinet was cubical in form, 2 feet on a side, with a side door. An observation window was built into the front, but this was normally kept covered to exclude stray light. The inside was painted white. Illumination was by nine 15-watt incandescent bulbs and four 17-inch Sylvania "Cool White" fluorescent tubes in the ceiling to simulate daylight. The lights were on for twelve hours a day

and were controlled by a time switch (Tork Time Controls Inc., Mount Vernon, N.Y.). Cooling was effected by means of a fan pulling room air through vents in the sides and out through slots in the top of the cabinet. The interior temperature of the cabinet rose to 75° F during the period of illumination and dropped to 65° - 70° F during the dark period.

Reagents. Unless otherwise specified, all reagents were analytical grade and obtained from the Fisher Scientific Co., Edmonton, Alta., or Montreal, P.Q. Such other reagents as were described in Section II were from the same suppliers and of the same grade as those named in that Section.

Bio-Rad AG2X-10 quaternary ammonium polystyrene anion exchange resin, 200-400 mesh, was obtained from Bio-Rad Laboratories, Richmond, Calif., and prepared for use according to the instructions given in the Beckman Model 120B Amino Acid Analyzer Instruction Manual (4).

In addition, a hydroponic nutrient medium of the following composition was used to grow seedlings under controlled conditions:

1.0 m$\underline{M}$ with respect to KH_2PO_4;

2.0 m$\underline{M}$ with respect to $MgSO_4$;

5.0 m$\underline{M}$ with respect to KNO_3;

5.0 m$\underline{M}$ with respect to $Ca(NO_3)_2$.

To each liter of this basal medium was added 1 ml of a micronutrient solution of the following composition:

0.1 mM with respect to Mo^{++};
0.3 mM with respect to Cu^{++};
1.0 mM with respect to Zn^{++};
1.0 mM with respect to Mn^{++};
1.0 mM with respect to BO_3^{---}.

(Adapted from Fergus (18)).

Biological materials. Germination of Lupinus luteus seeds was begun in darkness on damp filter paper and at this stage they were washed daily to remove any traces of microorganisms that may have started to grow on their seed coats. When the seed had swollen it was transferred to the growth cabinet described above. Thus only those seeds that were going to germinate at all were used.

Since it was necessary to recover the root systems intact for alkaloid analyses on weighed plants the swollen seeds were grown in 50-ml beakers filled with quartz chips 2-3 mm in diameter, which neither adhered to nor damaged the roots, while being small enough to retain a surface coating of nutrient solution by capillary action. The nutrient solution described above was changed daily in all the beakers.

Procedure. Seedlings were prepared for amino acid analysis by the following method, adapted from the procedure given in the Instruction Manual for the instrument (4):

1. About three grams of wet tissue was ground in 3 x 10 ml of 1% picric acid, and the resultant suspension was centrifuged immediately after each grinding;

2. The pooled supernatants were passed through a 1-inch diameter column of Bio-Rad AG2-X10 to remove excess picric acid;

3. The resin bed and the walls of the tube were washed with 5 x 3 ml of 0.02 N HCl;

4. The effluent and washings were concentrated to 1 ml in a flash-evaporator;

5. The concentrate was transferred to a small glass tube, the volume being kept below 3 ml. At this stage, overnight frozen storage was possible;

6. The pH was brought to 7.2 - 7.5 with 1 N NaOH (about ten drops); 0.2 ml of fresh 0.5 M Na_2SO_3 was then added. The pH was readjusted if necessary and the solution was allowed to stand in air for four hours;

7. The pH was adjusted to 2.0 - 2.2 with 1 N HCl (about ten drops);

8. The solution was adjusted to exactly 5.0 ml and stored in the freezer;

9. Two-ml aliquots were used for the analysis.

For any one alkaloid analysis, sufficient seedlings or seeds were used to make a total of about three grams of tissue. This varied from ten (with the swollen seeds) to three (with three-week-old seedlings). The characteristics of the seedlings over the germination period are shown in Table V along with the designations used for each of eight well-defined growth stages chosen for analysis.

Table V. Characteristics of L. luteus seedlings over period of germination

Designation	Developmental stage	Age (days)	Fresh wt. (grams)
A	Seed swollen	0.0 (defined)	0.32
B	Root tip visible	1.0	0.31
C	Germination complete	1.9	0.35
D	Cotyledons green	4.1	0.53
E	Cotyledons open	6.8	0.60
F	Primary leaves open	9.3	1.08
G	Secondary leaves open	14.4	1.17
H	Tertiary leaves open	20.3	1.37

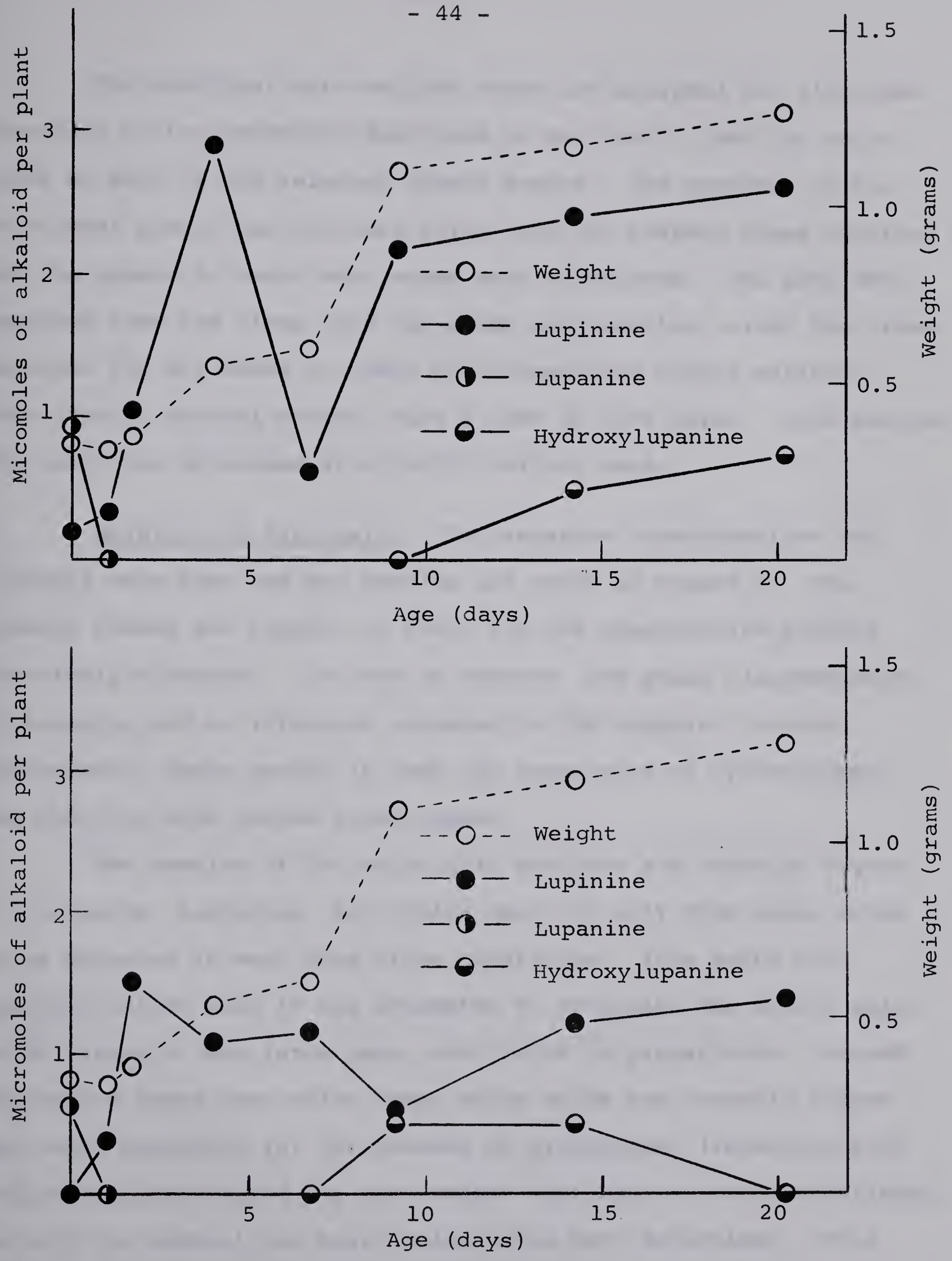
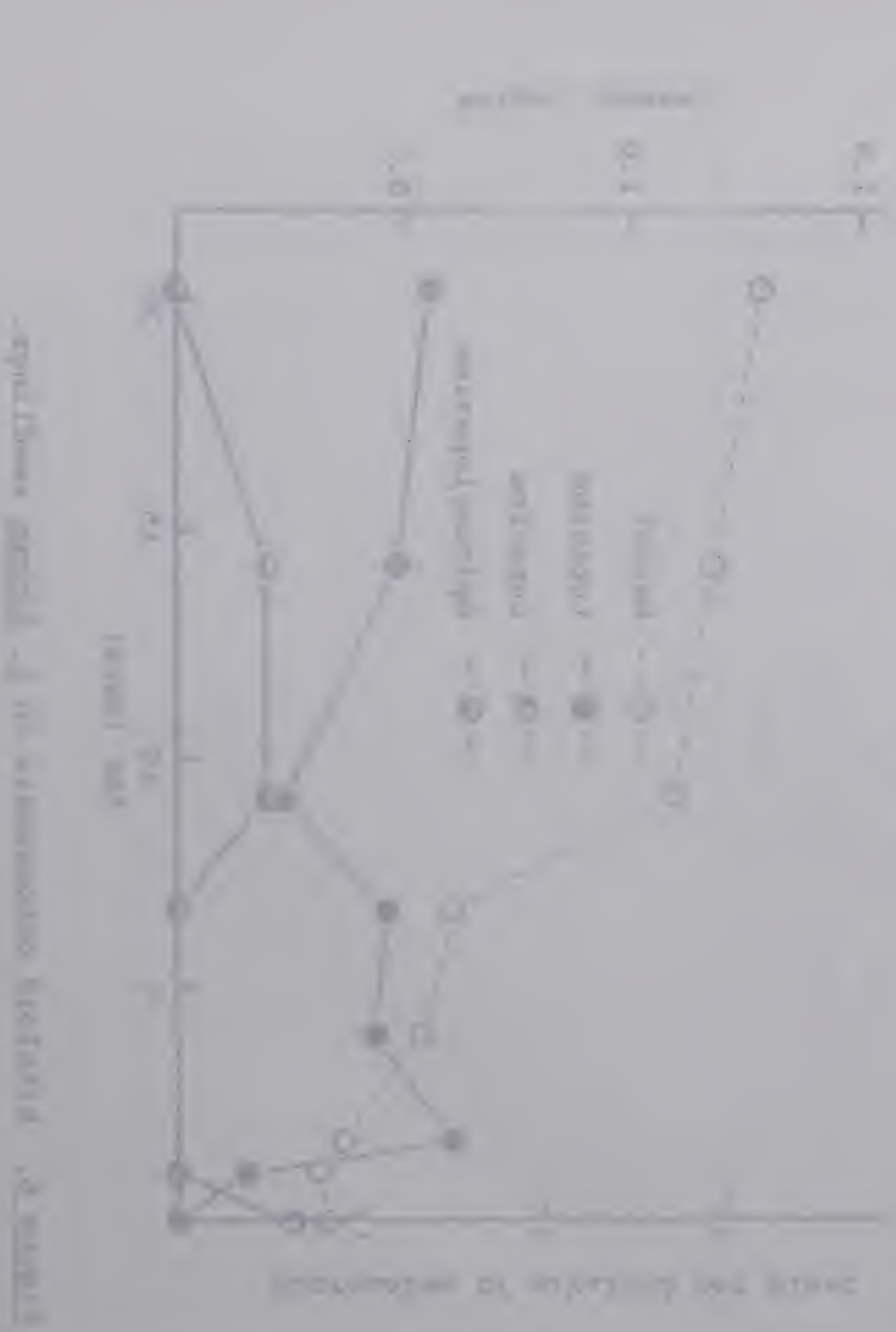


Figure 6. Alkaloid ontogenesis in L. luteus seedlings.



The seedlings were weighed fresh and analyzed for alkaloids according to the procedure described in Section II, and for amino acids at each of the selected growth stages. The progress of the individual plants was followed daily, and the average times required for the plants to reach each stage were calculated. The ages were reckoned from the times when the seeds were swollen, since the times required for dry seeds to reach this stage were highly variable (two days to several months, with a mode of five days). Once swollen, the seedlings developed at a fairly uniform rate.

Results and discussion. Two separate determinations for alkaloid were done and the results are shown in Figure 6. The general trends are similar in both, but the quantitative effects are widely divergent. In both we observe the rapid disappearance of lupanine and an irregular increase in the lupinine content. Furthermore, there occurs in both the appearance of hydroxylupanine when the true leaves first appear.

The results of the amino acid analyses are shown in Figure 7. Arginine, histidine, and lysine were the only free amino acids to be detected in more than trace quantities. (The amino acid analysis failed when it was attempted to determine the acidic amino acids, since a very large peak, attributed to glutathione, covered the entire range over which these amino acids are normally eluted. The usual procedure (4) for removal of glutathione (reduction with sodium sulfite) failed for some reason that has not been established, so only the neutral and basic amino acids were determined. This

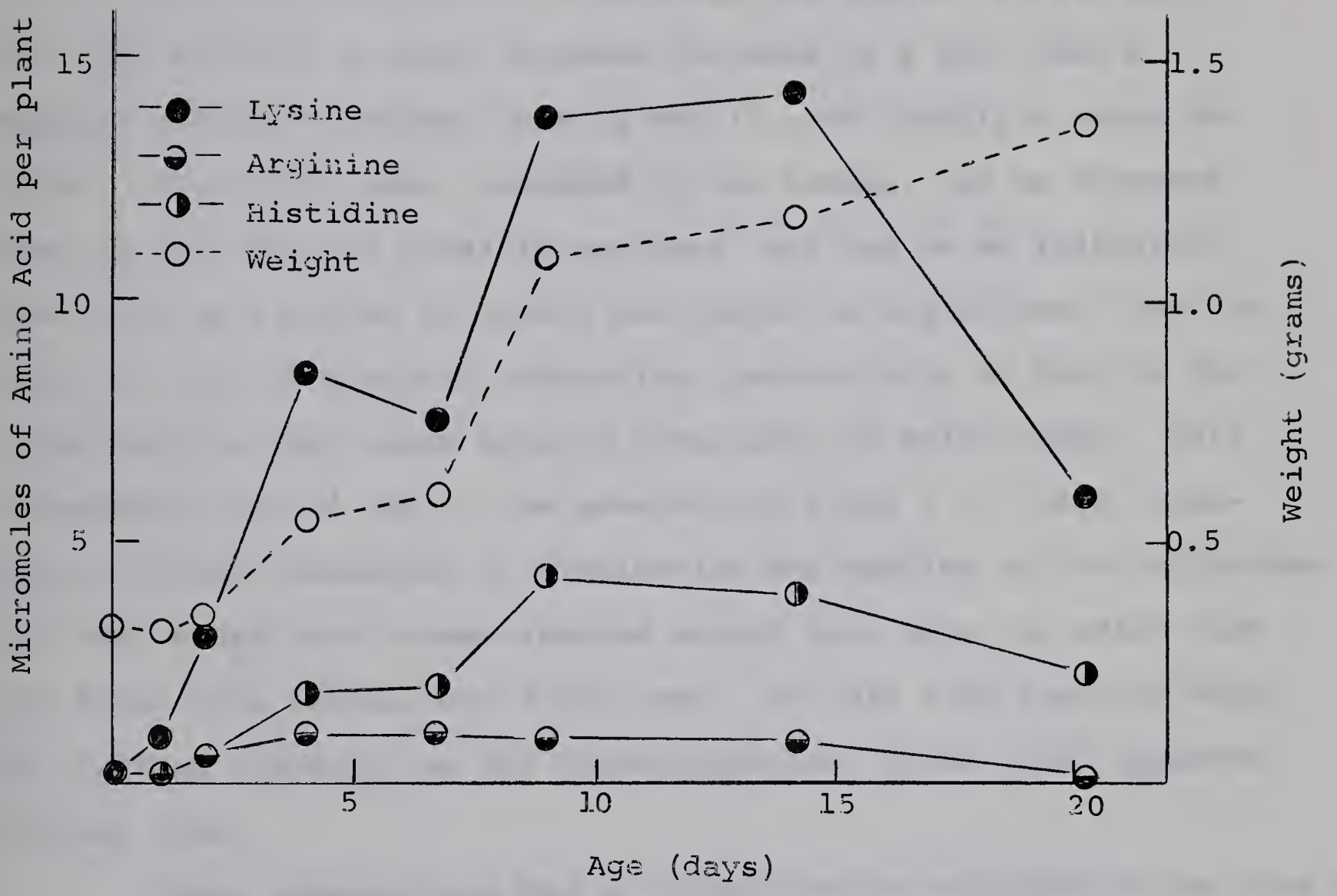


Figure 7. Amino acid ontogenesis in L. luteus seedlings.

was regarded as being of little consequence for this study, since the basic amino acids are those that have usually been associated with the metabolism of the alkaloids).

It will be observed that the progress curves for all three of the amino acids arginine, histidine, and lysine, follow much the same pattern: a rapid increase followed by a dip, then a maximum somewhere between Days 11 and 13, and finally a sharp decline. This early peak, followed by the trough, can be observed also in both sets of alkaloid analyses, and may be an indication that this is a period of growth that might be significant from the point of view of alkaloid metabolism, particularly in view of the relationships that exist between alkaloids and amino acids. This interesting period lay in the general age range 2 - 7 days, somewhere between completion of germination and opening of the cotyledons. The next significant stage started around nine days, by which time the first true leaves were fully open. At this time lupinine began to increase steadily, as did hydroxylupanine, which first appeared at that time.

These observations cast a certain amount of doubt on the idea that alkaloids are mere end-products of metabolism. Birecka *et al*. (11) obtained similar results for older plants at flowering and seed-pod formation, where high physiological activity might be expected. Both sets of observations indicate that the most rapid alkaloid metabolism takes place when the plant is physiologically most active, and suggest that alkaloids may play a more active role in the physiology of the plant then has hitherto been suspected.

IV. METABOLIC STUDIES IN VITRO

1. Introduction

All but one of the metabolic studies reported for the lupine alkaloids have been performed *in vivo*. The one exception is Nalborczyk's study of a crystalline (but nevertheless impure) lupanine dehydrase prepared from *L. albus* (54).

In vivo studies have the advantage of indicating the kinds of process that take place under the normal conditions of the plant. They are, however, limited in the extent to which they can give insight into the mechanisms involved in the reactions being investigated; they do not tell us what parts of the cell are involved, nor how far these reactions are influenced by other metabolic and physiological processes going on at the same time.

Consequently various preparations of *L. luteus* var. *Romulus* were tested to find out whether they metabolized alkaloids. A preliminary study was done using stem slices as the biological material to check whether alkaloid metabolism could be detected at all.

When stem slices *were* found to metabolize alkaloids, attention was concentrated on chloroplasts for the following reasons: (a) Birecka and Żebrowska (12) observed that in *L. luteus* and *L. albus* diurnal fluctuations took place both in the total alkaloid content and in the ratios among the contents of different alkaloids. This suggests an association of alkaloid metabolism with light and, by extension, with chloroplasts, the site of most

photobiochemical processes in the plant; (b) Kazimierski and Nowacki (28) concluded from grafting experiments among several different varieties of both sweet and bitter lupines that lupine alkaloids are synthesized exclusively in the green parts of the plant; this observation was verified in part by Birecka et al. (10) when they demonstrated alkaloid synthesis in derooted lupines; (c) White and Spencer (89) discovered from histochemical studies that the alkaloids of L. luteus are present in the highest concentrations in the vacuoles of those cells that contain the largest numbers of chloroplasts.

From the above facts it seemed likely that lupine alkaloid metabolism was at least partly associated in some way with the chloroplasts. Furthermore, from the results of the studies on the ontogenesis of the alkaloids (Section III) the most active stage of metabolic activity in young plants (seedlings up to three weeks old) seemed to close to the time when the first true leaves were opening. This stage was used for the preparation of the chloroplasts.

2. Experimental

Apparatus. A Warburg apparatus by B. Braun Apparatebau, Melsungen, German Federal Republic, made for Bronwill Scientific Co., Rochester, N.Y., was modified by removing the heating element and installing three 75-watt floodlights under the transparent bottom of the water-bath. A Plexiglass heat shield was installed between the lights and the water-bath. Since this only partially overcame the heating problem the temperature of the water was

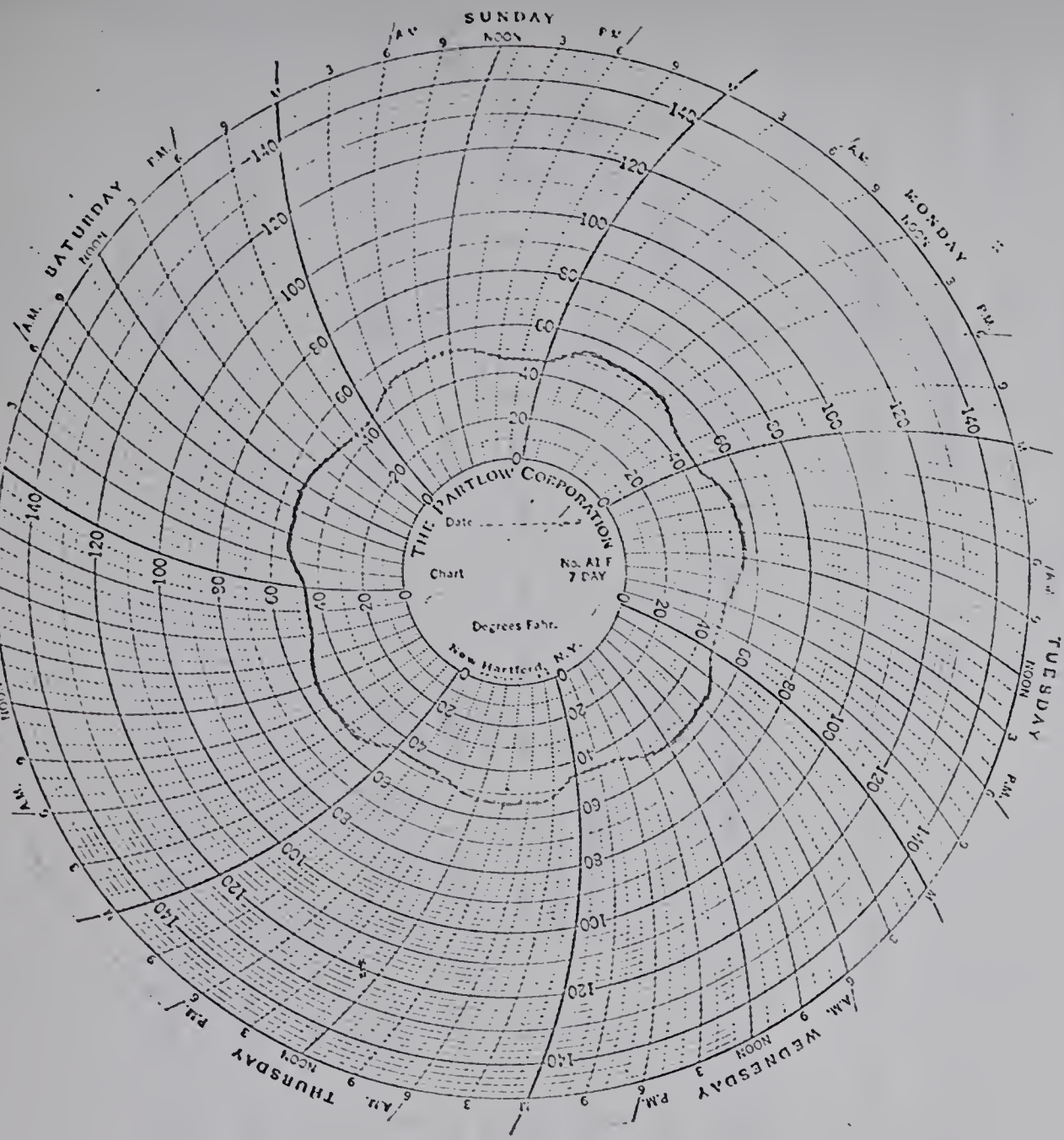
kept close to 27° C by addition of ice as necessary in the absence of a controlled refrigeration system. A metabolic shaker by Labline Inc., Chicago, Ill., was used for metabolic studies in darkness.

Other apparatus was as described in previous Sections.

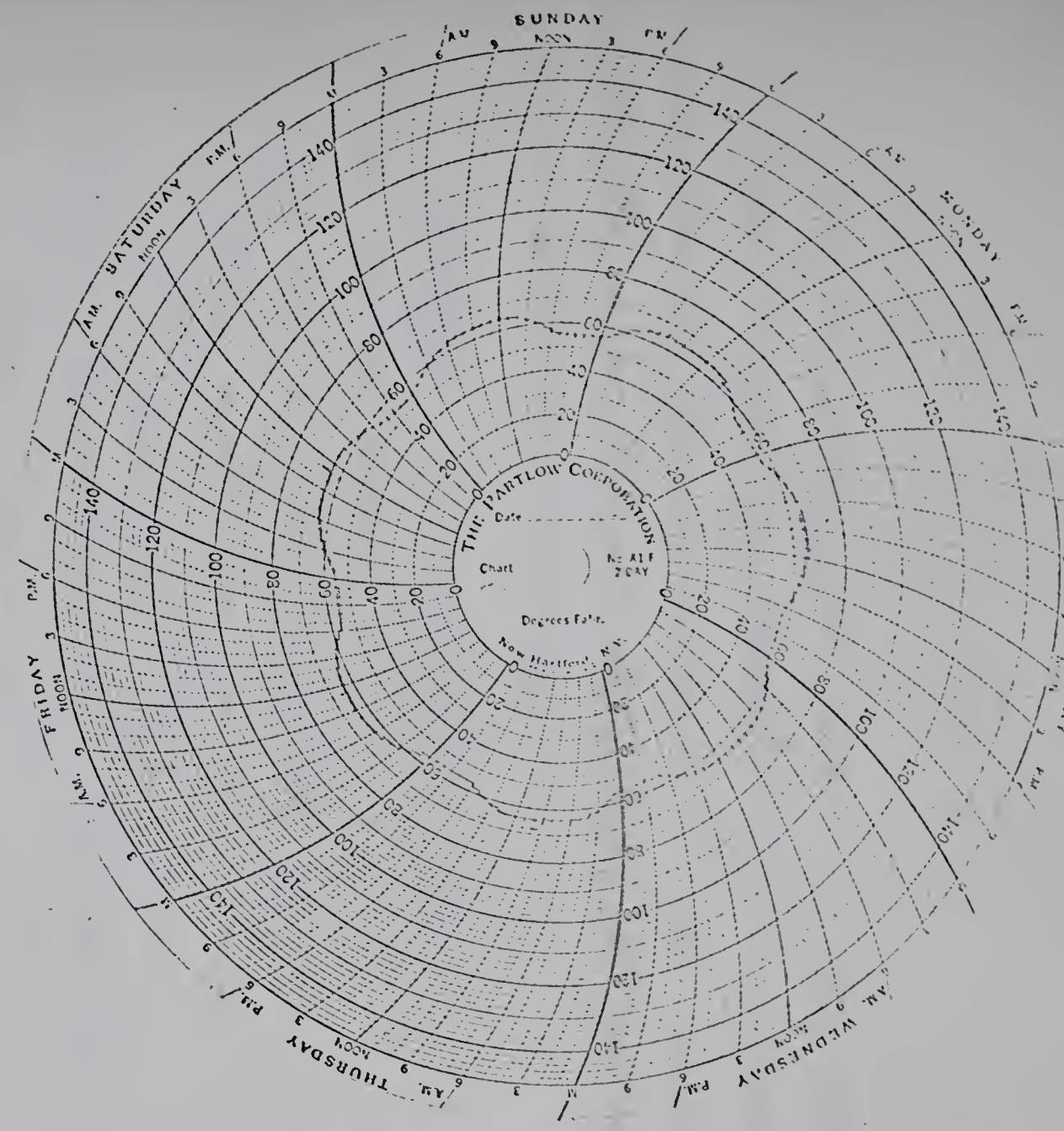
Reagents. Lysine, cadaverine, and pipecolic acid were obtained from Mann Research Laboratories Inc., New York, N.Y.; enzyme cofactors (AMP, pyridoxal phosphate, NADP, and NADPH) were obtained from the Sigma Chemical Co., St. Louis 18, Mo.; a sample of asymmetrical tripiperideine was obtained as a gift from Dr. Y.L. Chow of the Department of Chemistry, University of Alberta. The tripiperideine was recrystallized five times from acetone before use. Such other reagents as have been mentioned previously were of the same grades and from the same suppliers as before. All others were obtained from the Fisher Scientific Co., Edmonton, Alta., or Montreal, P.Q., and were of analytical grade.

Biological materials. Stem slices were from field-grown plants at the stage just prior to flowering.

Plants for chloroplast production were grown in sand under controlled conditions in a growth chamber (Coldstream Refrigeration Mfg. Ltd., Winnipeg 21, Man.). The wet- and dry-bulb temperature profiles of the chamber were as shown in Figure 8; the relative humidity was set at 55%; and the lights were adjusted so that the first of three banks was on from 5:00 a.m. to 5:00 p.m., the second from 6:00 a.m. to 6:00 p.m., and the last from 7:00 a.m. to 7:00 p.m. This was intended to give an approximate simulation of dawn and dusk.



(a) Wet-bulb



(b) Dry-bulb

Figure 8. Wet- and dry-bulb temperature profiles for Coldstream growth cabinet for a typical week.

When the seedlings had reached the stage at which they were to be used for chloroplast production they were harvested and stored in darkness for forty-eight hours at 2° - 4° C in order to reduce the starch content, since starch granules are difficult to remove during isolation of chloroplasts.

Procedure. (i) Stem slices. Sets of 25 stem slices, 1-2 mm thick, each set weighing a total of 5.0 ± 0.2 g, were incubated with each of the following substrates: sparteine, lupinine, lupanine, hydroxylupanine, oxolupanine, lysine, cadaverine, and pipecolic acid, all 0.01 M in 0.1 M potassium phosphate buffer, pH 6.50. In each case 10 ml of the substrate solution was used and incubated with the slices in a 50-ml Erlenmeyer flask in a shaking water bath at room temperature (27° C) for 24 hours. A control was used, with buffer replacing the substrate solution. In this preliminary run no attempt was made either to exclude daylight or to provide artificial illumination.

After incubation the supernatant was decanted off and stored in the frozen state until analyzed. Alkaloids in the slices were determined according to the usual procedures. The supernatant was analyzed in a more direct manner as follows: five drops of 10 N NaOH were added to each and the small precipitate thus formed was centrifuged down after mixing. The resultant yellow solution was extracted with 5 x 2.5 ml of chloroform. Some emulsion was formed but this was broken by centrifugation. After this the analysis was carried out exactly as if the resin had been used, except that the

alkaloids were not eluted from the paper for quantitative estimation. Since this was a preliminary investigation designed to indicate broad trends only, simple visual inspection of the stained chromatograms was used to give a rough indication of the quantities of any products yielded.

(ii) Chloroplasts. In some preliminary experiments chloroplasts were isolated in 0.35 M NaCl by differential centrifugation at various g values (2), but for all later experiments (with exceptions to be noted in Section V) the following method was employed: 500 ± 200 grams of derooted seedlings at the stage just prior to the appearance of the first true leaves were ground in a chilled mortar with cold 20 - 40 mesh acid-washed sand and their own weight of a grind buffer of the following composition: 0.35 M with respect to sucrose and 0.01 M with respect to NaCl, dissolved in 0.2 M Na_2HPO_4 - 0.1 M citric acid buffer, pH 4.50 (27). The mass of crushed tissue was squeezed through five layers of cheesecloth and the juice centrifuged at 100 g for three minutes to remove debris. (The first time this method was attempted this centrifugation was carried out at 755 g for ten minutes, but this brought down the chloroplasts as well; the second time 200 g for ten minutes was used, but this had the same effect. Thereafter 100 g for three minutes was found to be satisfactory.) The supernatant was decanted and spun at 1475 g for twenty minutes. The pellet was resuspended in the same volume of grind buffer as had been used for the initial centrifugation and spun down for a second time at 1475 g for twenty minutes. If, before resuspension, the pellet was layered, only the top (dark

green) layer was used, this being carefully scraped free with a plastic spatula, then suspended in the grind buffer. The second spin always brough down a visually uniform pellet; this pellet was suspended in 15 - 25 ml (depending on the number of samples required) of 0.35 M NaCl for use. All the above operations were carried out between 0^{o} and -5^{o} C.

Microscopic examination of the chloroplasts obtained by isolation in NaCl or in buffer indicated that fairly extensive disruption had taken place. Since it is extremely difficult (33) to obtain intact chloroplasts in large yield, the broken ones were used in the hope that disruption would not destroy their activity, but it was nevertheless borne in mind that results obtained with such preparations must be treated with some reserve lest disruption caused abnormal reactions to take place.

Reaction mixtures were made up as follows: 2.0 ml of chloroplast suspension was added to a solution consisting of 2.0 ml of 0.01 M substrate in 0.1 M potassium phosphate buffer, pH 6.50, and 1.0 ml of a cofactor mixture made by mixing equal volumes of 5.0 mM AMP, 5.0 mM pyridoxal phosphate, 5.0 mM NADP, and 1.0 mM NADPH, all made up in 0.1 M potassium phosphate buffer, pH 6.50, which was itself 1.0 mM with respect to $MgSO_4$ and 5.0 mM with respect to $NaHCO_3$.

The cofactors included were chosen because they are associated in some way with transamination, deamination, and decarboxylation reactions, reactions that might be expected to play some part in alkaloid metabolism.

Various controls were prepared by (a) substituting 0.35 M NaCl for the chloroplast suspension; (b) substituting 0.1 M potassium phosphate buffer, pH 6.50, for the substrate solution; and (c) substituting the buffer just named, with, and without, $MgSO_4$ and $NaHCO_3$, for the cofactor mixture.

Reactions were carried out by incubating the mixtures at 27° C for three hours in 10-ml Erlenmeyer flasks in a covered shaking water-bath if the reactions were to be studied under conditions of darkness, and in the modified Warburg if the effects of light were to be studied. The three-hour reaction period was chosen originally because it was long enough to permit the slow alkaloid reactions to proceed reasonably far, but not long enough to cause difficulties due to confusing side reactions. Some longer reaction times (18 - 24 hours) were in fact used in a number of experiments, but this was found to make no apparent difference whatsoever in the results obtained, so the precaution of limiting reaction times was actually superfluous.

After incubation the reaction mixtures were transferred to clinical centrifuge tubes and the reaction vessels washed out with 2.0 ml of 0.2 M potassium phosphate buffer, pH 6.50. (0.2 M buffer was used to bring the overall molarity of the buffer in the mixture to 0.1 M.) The chloroplasts were spun down and the supernatant decanted for analysis in the manner normally employed for a buffered plant extract. The very small chloroplast pellet was washed with a little 0.1 M buffer and the washings were added to the supernatant. Zero-time controls were prepared by adding the substrate directly

to the buffered resin instead of including it in the reaction mixture; after the substrate had had time for adsorption, a sample of centrifuged, substrate-free reaction mixture was passed through the column immediately prior to elution with acid; after this, processing was exactly the same as for the other samples.

Each experiment was duplicated in a parallel run using another sample of chloroplasts from the same preparation, this sample having been autoclaved at 121° C for seven minutes.

Results and discussion. (i) Stem slices. Table VI compares the metabolic activities of the slices towards the various substrates. In each case, the nature of the substrate used is compared with the alkaloid composition of (a) the slices; and (b) the supernatant, after incubation. Inspection of the table reveals some differences between the results from the two. In the supernatants only one obvious change has taken place, presumably by a reaction that took place in the slices - the apparent conversion of some cadaverine to sparteine. In the slices the observed changes (allowing for the fact that the slices themselves contained some sparteine to begin with) are fairly numerous. In particular it would appear from the analyses of the slices that there is a dynamic equilibrium set up between lupinine and lupanine, with the balance heavily in favour of the lupinine. This observation is contrary to all previous reports on the general trend of lupine alkaloid metabolism (7, 8, 9, 60), in which degradative reactions have never before been reported, except in bacteria (13, 26, 52, 83, 84). This conclusion is admittedly equivocal, but it is consistent with the result from the ontogenesis studies, in which lupinine was observed to increase and lupanine to decrease during germination. The fact that

Table VI. Alkaloids recovered from lupine stem slices and from substrate solutions after incubation of slices with substrates

Nature of Products	Nature of Substrate								
	Sp	Li	La	HOLa	OLa	Lys	Cad	Pip	Bl.
From slices:									
Sp	++++	++	++	++	+	++	++	+	+
Li		+++	+++	+	+	+	+		
La			+	+					
HOLa				+++					
OLa					+				
Unk.					+				+
From solutions:									
Sp	++++						++		
Li		+							
La			+++						
HOLa				+++					
OLa					+++				
Unk.									

+ signs indicate approximate quantitative relations between alkaloid products recovered (as listed to left side of table). Lysine, cadaverine, and pipecolic acid were not estimated. Abbreviations used: Sp - sparteine; Li - lupinine; La - lupanine; HOLa - hydroxylupanine; OLa - oxolupanine; Bl. - blank; Unk. - unidentified alkaloid.

the composition of the supernatants was little changed was probably because of a slow rate of diffusion of reaction product out of the tissue back into the solution.

(ii) Chloroplasts. A preliminary survey run was carried out using each of the following as substrates: sparteine, lupinine, lupanine, hydroxylupanine, lysine, cadaverine, pipecolic acid, and tripiperideine, in the reaction mixtures outlined above. Inspection of the chromatograms after completion of the run showed that, of these substrates, only sparteine seemed to have undergone any change between zero time and the end of the three-hour incubation period. It appears, therefore, that stem slices from older plants do not metabolize lupine alkaloids in the same way as do chloroplasts from young seedlings. The change that took place in sparteine was a slight reduction in the quantity of sparteine recovered, with the concomitant appearance of a new, unidentified substance staining like an alkaloid with Dragendorff-Munier reagent, reacting like an alkaloid with modified (Section II) Reifer reagent, and which had an R_{sp} (HCl Bu-Tol) of 0.50. This R_{sp} corresponds with none of those observed for any of the other alkaloids used as substrates in any of the present studies.

Most surprising of all, however, was the fact that sparteine incubated with autoclaved chloroplasts also underwent this conversion between zero time and three hours. Several repetitions of the experiment yielded identical results. Further checks on the authenticity of this conversion yielded the following information:

(a) Neither fresh nor autoclaved chloroplasts showed any sign of the new alkaloid unless they had been incubated with sparteine;
(b) Sparteine incubated with saline, but no chloroplasts, did not undergo this change, whether or not any of the cofactors (organic or inorganic) was present, and whether or not it was illuminated during the incubation period. Therefore chloroplasts and sparteine wereboth required for this product to be formed.

V. ISOLATION AND CHARACTERIZATION OF A HEAT-STABLE FACTOR PRESENT IN CHLOROPLASTS AND REACTIVE TOWARDS SPARTEINS

1. Introduction

In Section IV was presented evidence that if sparteine is incubated with chloroplasts from L. luteus, some of the sparteine is converted to another substance that stains like an alkaloid with Dragendorff-Munier reagent. Furthermore, autoclaving the chloroplasts did not cause loss of this property, so whatever factor was responsible for it must have had considerably greater heat-stability than many substances of biological origin. It was therefore almost certainly not an enzyme, although some enzymes can be boiled without loss of activity, for example ribonuclease (17). Besides, it is not clear from the evidence presented thus far whether this factor was a catalyst or a simple reactant.

A number of experiments was therefore carried out to further characterize this factor.

2. Apparatus and reagents

Apparatus. Infra-red spectra were run on a Perkin-Elmer 421 Grating Spectrometer by the Perkin-Elmer Corporation, Norwalk, Conn., and mass spectra were run on a Metropolitan-Vickers MS-2H (modified) Mass Spectrometer by Associated Electrical Industries Ltd., Manchester, England. The X-ray fluorescence studies were done on a Norelco Universal Vacuum X-ray Spectrograph by Philips Electronic Instruments, Mount Vernon, N.Y. Sonication was carried

out in a Raytheon Sonic Oscillator by Raytheon Manufacturing Co., Waltham, Mass.

Reagents. AG1-X4 quaternary ammonium polystyrene anion exchange resin was obtained from Bio-Rad Laboratories, Richmond, Calif., and was prepared for use by equilibrating it overnight with concentrated aqueous ammonia, followed by a demineralized water wash until the washings were neutral. AG50W-X sulfonic acid polystyrene cation exchange resin was obtained from the same source and was used directly after washing with deionized water.

All reagents named in previous Sections were of the same grades and from the same suppliers as those named in the relevant Sections. All others were analytical grade reagents from the Fisher Scientific Co., Edmonton, Alta., or Montreal, P.Q.

Biological materials. Chloroplasts were prepared as described in Section IV, except in some later experiments where, for reasons made clear in the text, 0.35 M NaCl was substituted for the sucrose grind buffer. Times of centrifugation and g values were unchanged. The plants for chloroplast production were grown and harvested under the same conditions as those used in Section IV, and were used at the same stage of development.

3. Preliminary characterization of the factor as to solubility and particle size

Procedure. A 5-ml aliquot of chloroplast suspension was sealed into a short length of dialysis tubing and dialyzed for a

total of 24 hours against three changes of suspension medium (0.35 M NaCl) prior to incubation with sparteine. Another 5 ml of suspension from the same preparation was stored for 24 hours at 2° C in its original saline suspension medium, then centrifuged free of chloroplasts. The saline supernatant was then incubated with sparteine. In each case 2.0 ml of suspension or saline supernatant was incubated for three hours at 27° C with 2.0 ml of 0.01 M sparteine sulfate in 0.1 M potassium phosphate buffer, pH 6.50, and a further 2.0 ml of each was used for zero-time analysis. This was done with both fresh and autoclaved chlorplasts.

Results and discussion. None of the zero-time analyses revealed any sign of a product. Nor did the three-hour analyses of the samples of sparteine incubated with dialyzed chloroplasts, but the chromatograms of the sparteine incubated with the supernatant from the centrifuged chloroplasts (whether fresh or autoclaved) showed a faint but quite distinct spot with the R_{sp} of the usual product.

From the above we can readily conclude that, if a single factor is involved, it is (a) soluble in 0.35 M NaCl; and (b) probably not a macromolecule. If, on the other hand, a mixture of factors is involved, the following are probably true: (a) all components of the mixture are soluble in 0.35 M NaCl; and (b) at least one of them is not a macromolecule.

4. Isolation and further characterization of the factor

Procedure. Chloroplasts were prepared in the usual way

from 250 - 500 g of green tissues from lupine seedlings, and the resultant pellet of chloroplasts was suspended in 25 ml of water (rather than saline) in order to lyse them. The suspension was stirred overnight in the cold, and further disruption was carried out by five minutes of sonication before centrifugation at 12,100 g for thirty minutes. The supernatant was autoclaved at 121° C for seven minutes to precipitate protein, which was then centrifuged off, and to inactivate any heat-labile factors that may have been present to interfere. The supernatant was then freeze-dried. This procedure usually yielded about 0.5 - 1.0 g of yellowish-white powder.

The following procedure was used to decide whether the powder was in fact active: 100 mg (corresponding to about 40 g of green lupine tissue) was dissolved in 4 milliliters of water. Two milliliters of this solution was mixed with 2.0 ml of 0.01 M sparteine sulfate in 0.1 M potassium phosphate buffer, pH 6.50, and incubated at 27° C for three hours. The remaining 2.0 ml was used for a zero-time control, and the two samples were analyzed in the usual way.

It remained to determine the nature of this factor. Several preliminary physical and chemical examinations were carried out: a 50-mg sample of the powder isolated from chloroplasts was submitted to X-ray fluorescence analysis; a 10-mg specimen was subjected to amino acid analysis; and the absorption spectrum of a 1% solution was obtained in the range between 180 and 800 mμ.

A fresh preparation of the factor was made from chloroplasts in the same manner as before, and after the freeze-drying stage it was redissolved in 10 ml of water, deionized with both resins in

order to remove charged impurities, and freeze-dried once again. About 9.5 mg of yellowish powder remained. This powder should therefore have been the purest sample of the factor as yet obtained. It was then subjected to infra-red analysis in the form of a Nujol mull, since it proved to be insoluble in the usual solvents employed for infra-red analysis.

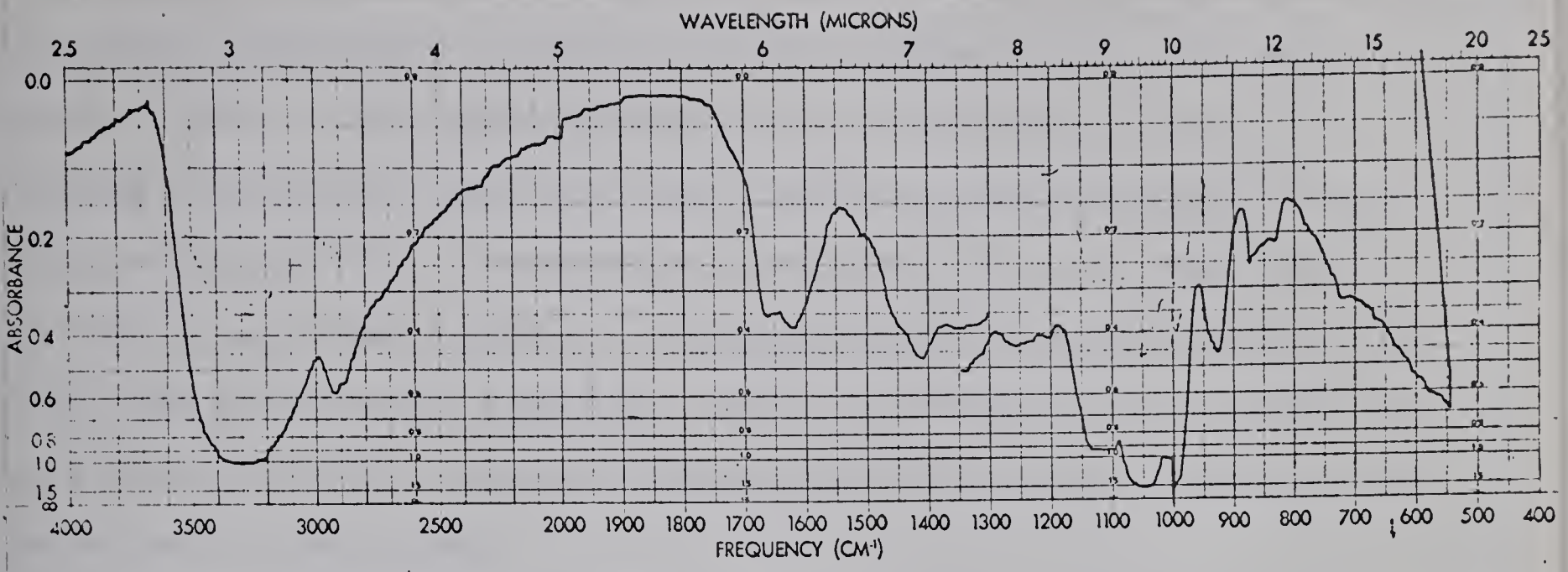
Results and discussion. The isolated powder was found to be in fact active, the zero-time control showing no sign of the product and the three-hour incubation run showing a partial conversion to the usual product.

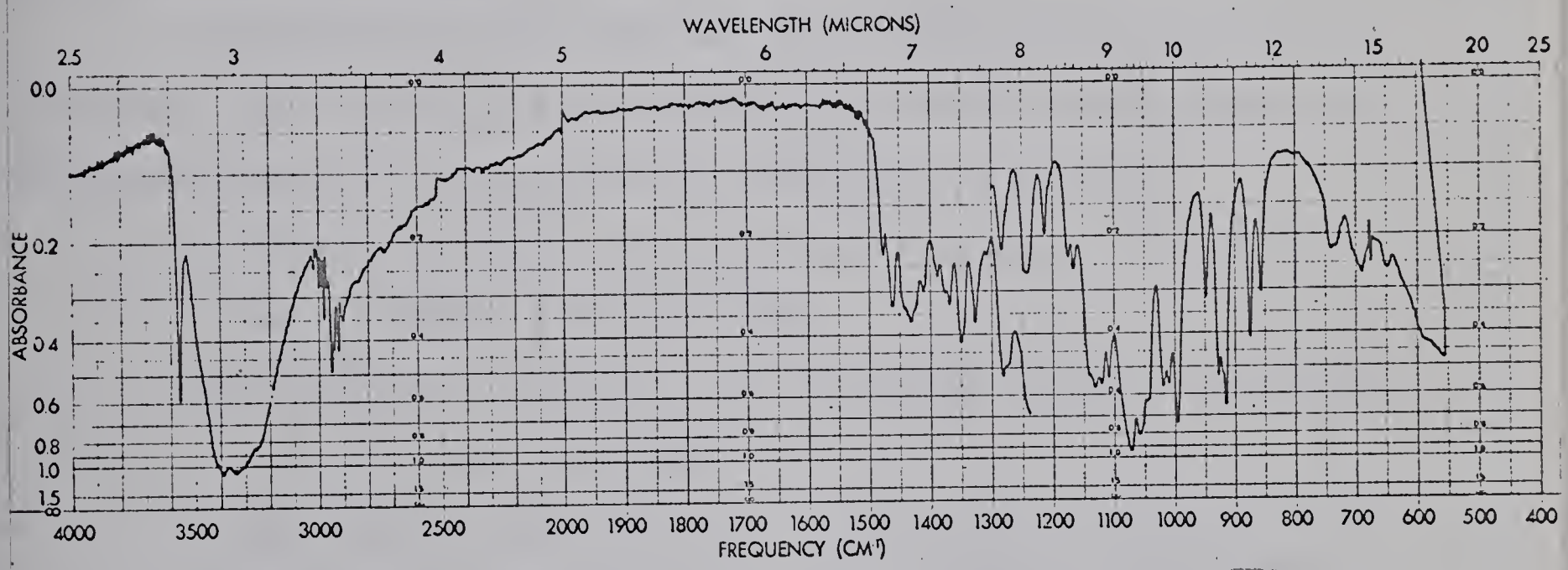
The X-ray diffraction studies showed that heavy metals were not present to an extent greater than one part in 10^5; the amino acid analysis showed that no amino acids were present in quantities greater than 0.2 mole in 10 mg of powder; and the spectrum of the 1% solution showed no absorption bands in the range 180 - 800 mμ.

The infra-red spectrum of the factor (Figure 9) revealed bands corresponding to carbonyls, ethers, and - particularly strongly - hydroxyls. This was highly suggestive of carbohydrate.

5. Electrostatic charges in the factor

Procedure. The following experiment was set up to determine what kind of electrostatic charges (if any) were borne by the factor; 250 mg of powder was dissolved in 10 ml of water. Five milliliters of this solution was freed of anions by passing it through a column of 20-50 mesh AG1-X4 resin in the hydroxyl form. The remaining 5 ml of the aqueous solution of the unknown compound was freed of





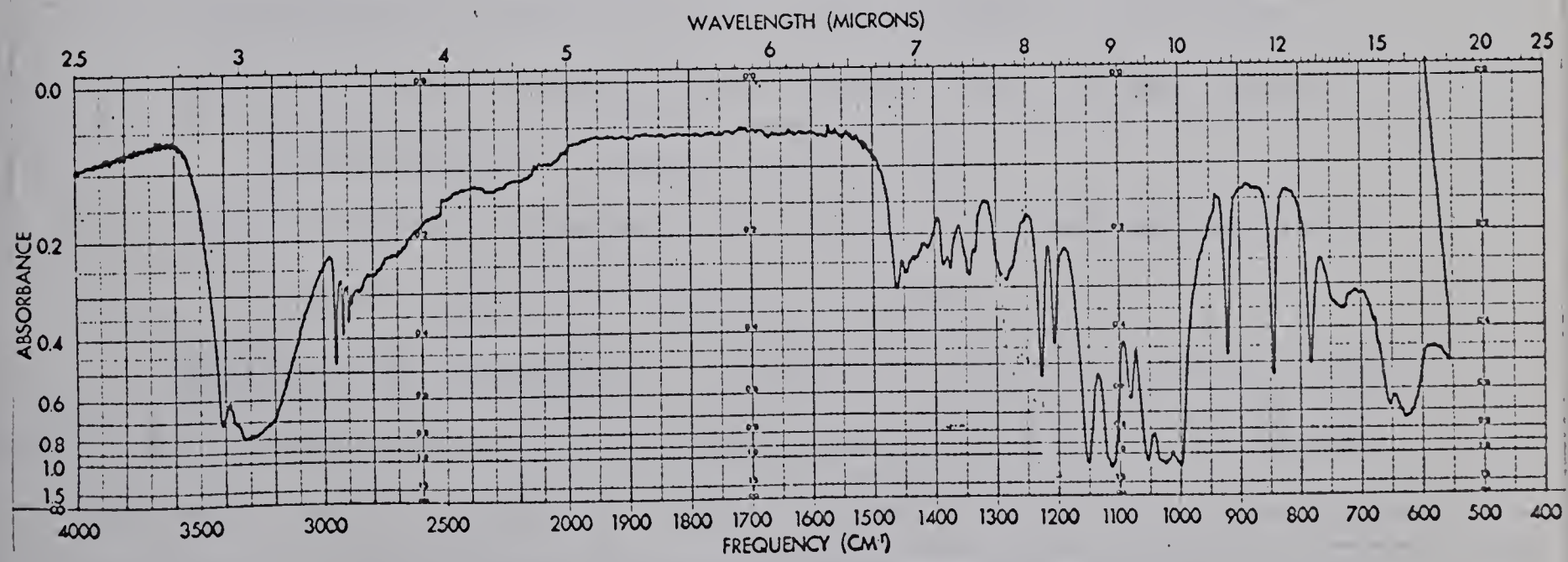


Figure 9. Infra-red spectrum of chloroplast factor (top), compared with that of sucrose (centre) and glucose (bottom).

cations by passing it through a similar column of 50 - 100 mesh AG50W-X resin in the hydrogen form. A 2.0-ml aliquot from the effluent from each column was used for zero-time analysis of activity, and 2.0 ml for a three-hour incubation. In each case 2.0 ml of 0.01 M sparteine sulfate in 0.1 M potassium phosphate buffer, pH 6.50, was used as the substrate. The 1 ml remaining from each 5 ml of solution from the unknown compound from chloroplasts was lost by retention on the column.

A second experiment was set up to extend the results from this one. The following six mixtures were used in each case, with the same volumes and experimental conditions as before:

(a) Sparteine alone (i.e. no factor present; merely 2.0 ml of water) for zero time;

(b) Sparteine incubated with untreated factor to determine the original activity;

(c) Sparteine incubated with factor freed of anions;

(d) Sparteine incubated with factor freed of cations;

(e) Sparteine incubated with factor that had had anions removed prior to removal of cations;

(f) Sparteine incubated with factor that had had cations removed prior to removal of anions.

Results and discussion. Analysis of the preliminary experiment with the deionized factor showed that deionization decreased the activity, as estimated by visual inspection of the chromatograms, only slightly. In the more detailed experiment two products were observed to be produced - the usual one and another with an R_{sp}

corresponding to that of lupinine. The degrees of conversion to the usual product decreased in the order b>c=d>e=f>a=0. In other words deionization of either kind decreased the activity slightly, and double deionization in either order decreased it slightly more. For the new product the conversions decreased in the order b>c=d>e=f=a=0; that is, single deionization of either kind decreased the activity slightly, and double deionization in either order destroyed it completely.

Therefore the factor appeared to bear slight amphoteric charges, and the factor for the new conversion to have somewhat greater charges.

6. Reaction of sugars with sparteine

Procedure. To test the hypothesis that the factor was a carbohydrate, each of the following sugars was made up to 0.5 M: glycoaldehyde, ribose, arabinose, galactose, glucose, glucosamine, maltose, and sucrose (i.e. one diose, two pentoses, three hexoses - including one amino sugar, and two disaccharides). This high concentration was chosen in order to exaggerate any reaction that might take place so that it could be more readily detected. Two milliliters of each solution was incubated with 3.0 ml of sparteine in the usual buffer in the same way as was the isolated factor itself. After incubation these mixtures were analyzed for alkaloid in the usual manner, as was a control with no sugar present.

Results and discussion. The completed chromatograms from the sugar-sparteine reaction mixtures showed that at least five

different reaction products staining with Dragendorff-Munier reagent were formed by reaction of sparteine with glycoaldehyde, and that at least two were formed from each of ribose and arabinose (sugars do not stain with Dragendorff reagent). In all three cases one of the spots had the same R_{sp} as the product formed from sparteine on incubation with chloroplasts. Furthermore, all of the sugars tested showed the formation of one reaction product with the same R_{sp} as the product formed when chloroplasts were used, although similarity of R_{sp} does not, of course, mean that all nine products are identical. It should be pointed out that cellulose does not seem to mediate this conversion, since the product does not appear on the (cellulose) paper chromatogram unless the sparteine has been pre-incubated with chloroplasts or a sugar.

Although no reliable quantitative results were obtained, it appeared that no more product was formed from 0.5 M sugars than from the much more dilute solutions prepared from chloroplasts. The most obvious type of reaction mechanism to account for this is some form of catalysis that allows an equilibrium ratio of sparteine and product to be formed. This is further borne out by the observation that incubation prolonged beyond the three hours normally used made no apparent difference to the degree of conversion attained.

7. Isolation, comparison, and partial characterization of reaction products from chloroplasts and sugar.

Procedure. A batch of chloroplasts was prepared in 0.35 M NaCl rather than in sucrose grind buffer, and made up to 20 ml in saline. This suspension was divided into ten two-ml aliquots, each of which was incubated for 24 hours at 27° C with 2 ml of 0.01 M

sparteine in 0.1 M potassium phosphate buffer, pH 6.50. (In earlier pilot runs it had been found that incubating the entire yield of chloroplasts with a large volume of sparteine in a single run yielded no product; the reason for this effect is unknown, but it was consistent.) The saline-isolated chloroplasts were used in order to make sure that the reaction being investigated was in fact due to the chloroplasts, and not to the sucrose in the grind buffer.

After the incubation period, two of the reaction mixtures were processed in the usual way for separation of the alkaloids from the other components of the mixture, and the alkaloids thus isolated from each mixture were dissolved in 1 ml of 0.05 M HCl. A 50-λ aliquot of each was chromatographed and the chromatogram stained to make sure the reaction had in fact taken place.

When a positive result was obtained from the above test the remainder of the reaction mixtures were processed and each was finally dissolved in 0.05 M HCl in the same way as were the first two. The HCl solutions were pooled and streaked on several sheets of Whatman #3MM chromatography paper for bulk separation of the product from the sparteine.

After development of the chromatograms, half-inch strips were cut off each and stained with Dragendorff-Munier reagent to locate the product. Using these strips as a guide, the bands containing the product were cut from the papers and eluted with 0.05 M HCl. The acid eluates were then basified with a slight excess of NaOH and extracted with chloroform. The chloroform extract was acidified with glacial acetic acid and evaporated to dryness in a stream of nitrogen. The sticky yellow residue thus formed was

washed in a few drops of methanol to yield about a milligram of yellowish-white crystals.

The above process was repeated using, instead of the chloroplast suspension, 0.5 M arabinose, this sugar being the one that usually gave the largest yield of product without excessive formation of other substances. This procedure again yielded about a milligram of yellowish-white crystals.

To check whether the two products were in fact the same, both were dissolved in chloroform and their ultra-violet spectra observed in the range 220-340 mμ. The chloroform was then evaporated off and the two samples were submitted to mass spectrometry.

Results and discussion. The ultra-violet absorption spectra of the two products were similar, both showing a peak at 244 mμ with an extinction coefficient of about 500 for a 1% solution with a 1-cm light path, and a pronounced shoulder at 262 mμ. The peak for the product from chloroplasts was somewhat broader than that for the product from the sugar. However, absorption in this range is fairly common among organic compounds, so this result, without further corroborating evidence, cannot be considered too significant, particularly since the cut-off point for chloroform itself is at 237 mμ.

The required corroborating evidence came in the form of the mass spectra of the two products (Figure 10). In both spectra the highest peak is in position 73 and the highest in the uppermost group of peaks (probably corresponding to the molecular weight of the substance) is in position 281. The spectra differ in detail, but the main groups of peaks are in the same places in both, although neither spectrum bears much resemblance to that of sparteine. It should be emphasized that blanks were run on eluates from paper chromatograms that did not contain any product, and that these gave no peaks whatsoever other than small common molecules such as water.

It therefore seems very likely that the product formed by reaction of sparteine with chloroplast suspensions from L. luteus var. Romulus is the same as that formed by reaction with arabinose - and conceivably with other sugars. The last point is, however, still an open one.

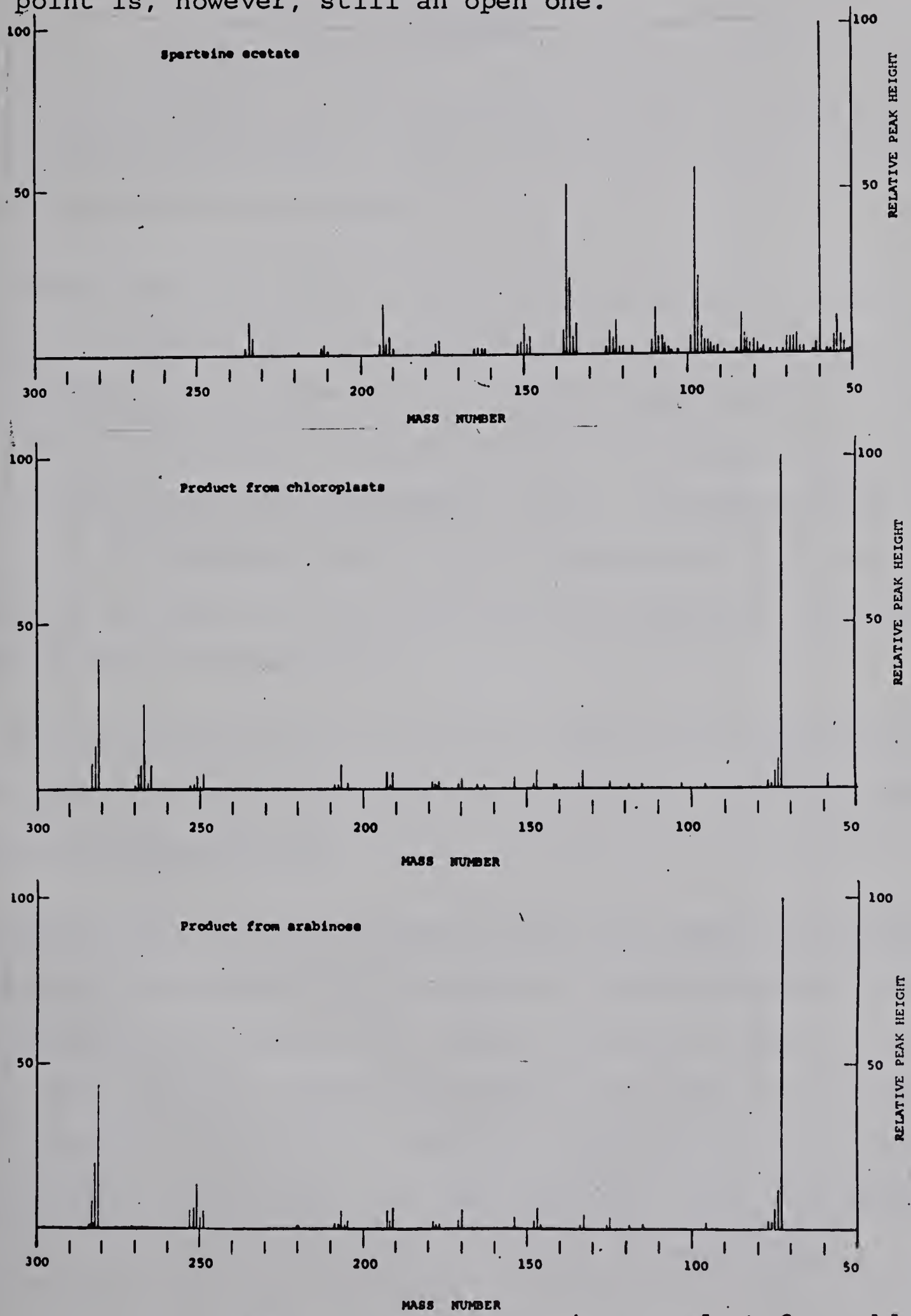


Figure 10. Mass spectra of sparteine, product from chloroplasts and product from arabinose.

SUMMARY

1. The quinolizidine alkaloids present in plant tissues were found to be readily extractable in near-quantitative yield by maceration of the fresh tissue with water.

2. These alkaloids could be removed from small volumes of tissue extract by passing the buffered extract through a column of Bio-Rex 63 phosphonic acid cation exchange resin. They could be eluted from the resin with acid and removed from the acid eluate by basifying it and extracting with chloroform. If the chloroform extract was acidified with glacial acetic acid and evaporated to dryness in a stream of nitrogen, the alkaloids could be separated from one another by paper chromatography.

3. The alkaloids could be estimated, after elution from the paper with acid, by a nephelometric technique based on one developed by Reifer and Niziołek (58).

4. Comparison of the ontogenesis of the alkaloids in L. luteus var. Romulus with the growth of the seedlings indicated that alkaloid metabolism in this species was somehow related to growth and development, since alkaloid metabolism seemed to be most active during rapid growth and at critical periods of physiological activity such as germination and at the time when the true leaves were beginning to take over from the cotyledons the task of metabolism.

5. Stem slices from L. luteus at a growth stage close to flowering, when incubated with lupine alkaloids, appeared to mediate certain interconversions between these alkaloids. Noteably, there seemed to be a dynamic equilibrium set up between lupinine and lupanine, with the balance in favour of the former.

6. Chloroplasts from lupine seedlings at a stage when the true leaves first appear converted sparteine to another, unidentified, substance reading like an alkaloid with Dragendorff reagent and Reifer reagent. This conversion still took place even after the chloroplasts had been autoclaved.

7. Experiments with washed chloroplasts, dialyzed chloroplast suspensions, and ion exchange resins indicated that whatever factor in chloroplasts was responsible for this reaction was a small molecule soluble in saline and free of electrostatic charges.

8. An isolation procedure based on the observed properties of the factor yielded about ten milligrams of active factor from the chloroplasts obtained from about five hundred grams of seedlings.

9. The infra-red spectrum of the isolated factor resembled that of a carbohydrate; this was consistent with the other observed properties of the material.

10. Various carbohydrates incubated with sparteine, under the same conditions as were the chloroplasts, yielded a product behaving chromatographically like that obtained when chloroplasts were used, and reacting in a similar manner with Dragendorff and Reifer reagents.

11. Larger-scale preparations of the product were carried out both with sugars and with chloroplasts, but even so, the yields were small (of the order of one milligram each). Preliminary examination of the two products by physical means yielded similar results, so the reaction observed with chloroplasts was probably in fact due to a sugar or sugars.

Suggestions for further investigation

Because of the unusual nature of this reaction, its mechanism will undoubtedly be of interest to the organic chemist, but before this mechanism can be investigated, the chemical nature of the product will have to be established.

Also, chloroplasts are the only subcellular structure to have been investigated in the manner described. What part, if any, is played by other fractions in this reaction, and in other aspects of alkaloid metabolism, is unknown. Furthermore, whether analogous reactions take place in other species with other alkaloids is an open question, as is that of the significance of the process in L. luteus itself, and in alkaloid metabolism generally.

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